Published in final edited form as:

Metabolomics. 2011 June 1; 7(2): 257–269. doi:10.1007/s11306-010-0249-0.

Stable isotope resolved metabolomics of lung cancer in a SCID mouse model

Teresa W.-M. Fan,

Department of Chemistry, University of Louisville, 2210 S. Brook St, Rm 348 John W. Shumaker Research Building, Louisville, KY 40292, USA

Department of Medicine, James Graham Brown Cancer Center, Clinical Translational Research Building, 505 S. Hancock St., Louisville, KY 40202, USA

Center for Regulatory Environmental Metabolomics, University of Louisville, 2210 S. Brook St., Louisville, KY 40292, USA

Andrew N. Lane,

Department of Chemistry, University of Louisville, 2210 S. Brook St, Rm 348 John W. Shumaker Research Building, Louisville, KY 40292, USA

Department of Medicine, James Graham Brown Cancer Center, Clinical Translational Research Building, 505 S. Hancock St., Louisville, KY 40202, USA

Center for Regulatory Environmental Metabolomics, University of Louisville, 2210 S. Brook St., Louisville, KY 40292, USA

Richard M. Higashi, and

Department of Chemistry, University of Louisville, 2210 S. Brook St, Rm 348 John W. Shumaker Research Building, Louisville, KY 40292, USA

Center for Regulatory Environmental Metabolomics, University of Louisville, 2210 S. Brook St., Louisville, KY 40292, USA

Jun Yan

Department of Medicine, James Graham Brown Cancer Center, Clinical Translational Research Building, 505 S. Hancock St., Louisville, KY 40202, USA

Teresa W.-M. Fan: twmfan@gmail.com

Abstract

We have determined the time course of [U-¹³C]-glucose utilization and transformations in SCID mice via bolus injection of the tracer in the tail vein. Incorporation of ¹³C into metabolites extracted from mouse blood plasma and several tissues (lung, heart, brain, liver, kidney, and skeletal muscle) were profiled by NMR and GC-MS, which helped ascertain optimal sampling times for different target tissues. We found that the time for overall optimal ¹³C incorporation into tissue was 15–20 min but with substantial differences in ¹³C labeling patterns of various organs that reflected their specific metabolism. Using this stable isotope resolved metabolomics (SIRM) approach, we have compared the ¹³C metabolite profile of the lungs in the same mouse with or without an orthotopic lung tumor xenograft established from human PC14PE6 lung adenocarcinoma cells. The ¹³C metabolite profile shows considerable differences in [U-¹³C]-

glucose transformations between the two lung tissues, demonstrating the feasibility of applying SIRM to investigate metabolic networks of human cancer xenograft in the mouse model.

Keywords

Stable isotope tracers; SIRM; SCID mouse; Metabolomics; Non-small cell lung cancer xenograft

1 Introduction

Stable isotope tracers have been widely used to map metabolic pathways and quantify fluxes in cells, tissues and organisms (Bak et al. 2007; Beger et al. 2009; DeBerardinis et al. 2007; Delgado et al. 2004; Fan et al. 1986, 1988, 2003, 2008, 2009b; Lane et al. 2008; Mancuso et al. 2005; Mason et al. 2007; Mendes et al. 2006; Thornburg et al. 2008; Vizan et al. 2005; Zwingmann and Leibfritz 2003). Although studies with cell cultures are best defined in terms of experimental control and cell-specific metabolism, they lack the ability to probe microenvironmental influences on cellular metabolism in a tissue context. This is crucial to translating understanding of metabolic functions in model cell systems into elucidating human metabolism in situ.

Deciphering the intricate web of human metabolic networks and their perturbations due to disease development or drug treatment is essential to the functional annotations of relevant gene (including single nucleotide polymorphism) and protein level changes. This task has been a difficult challenge, which cannot be resolved fully by total metabolite profiling alone. This is because a given metabolite such as glutamate can participate in many different reactions (e.g. >200, M. Arita, personal communication) such that its total concentration cannot discern changes in all—or even a few—reactions. Even if the concentration of various metabolites in a given pathway is known, it is still difficult to deduce which are the perturbed reactions in the pathway without ambiguity.

To achieve such an understanding, the use of labeled tracers is required. For example, the time course changes in normalized total lactate concentration of neurons induced by LiCl were opposite to the normalized concentration of triply ^{13}C labeled ($^{13}\text{C}_3$) lactate (Fig. S1, from Fan et al. (2010)). Based on the total lactate concentration alone, one would conclude that glycolysis in neurons was inhibited by LiCl. However, with the use of uniformly ^{13}C -labeled glucose ($^{13}\text{C}_6\text{-Glc}$), the enhanced production of $^{13}\text{C}_3$ -lactate by LiCl-treated neurons was evident, which requires an opposite interpretation, i.e. glycolysis was instead stimulated by Li. By combining labeled tracers with stable isotope-resolved metabolomic (SIRM) analysis, it is now possible to reconstruct multiple metabolic pathways based on numerous labeled metabolite patterns and to ascertain the impact of drug treatment or disease state on both targeted and unsuspected pathways (Fan et al. 2005, 2009b, 2010). In essence, the SIRM approach “encodes” each metabolite with pathway-specific dynamics.

Although tracer studies can—and have been—performed directly on human subjects (Fan et al. 2009b; Lane et al. 2009a; Mason et al. 2007), the degree of experimental control and sampling options are extremely limited due to confounding factors such as lifestyle and ethical concerns. In the past, radiotracers were extensively employed in metabolic research in animal models. However, they are unsuited for SIRM due to the general non-detectability of radiotracers by NMR, and their hazardous nature which places severe operational constraints for MS. Moreover, they are generally unsuited for in situ human studies. A few studies have explored the use of stable isotope tracers in investigating metabolic perturbations in animal models in response to drug or other treatments (Beger et al. 2009; Artemov et al. 1998; Bhujwalla et al. 1994). To translate detailed model cell findings to

understanding of human cancers in situ, it is generally accepted to employ xenograft animal models that more closely approximates the complexity of human tissues, while permitting a wider range of experimental and sampling protocols. The severe combined immunodeficient (SCID) mouse is one such model that enables transformed human cells to be established as xenograft subcutaneously or orthotopically in compatible organs. They are commonly used to investigate the tumorigenicity of human tumor cells and their response to drugs in preclinical studies (PaineMurrieta et al. 1997; Richmond and Su 2008).

The small size (e.g. 20 g) and high metabolic activity of SCID mice hold certain advantages for SIRM, i.e. small amounts of tracers and shorter duration required for extensive labeling in tissues or biofluids. We have previously determined the optimal sampling times for human NSCLC patients following $^{13}\text{C}_6\text{-Glc}$ infusion i.v. (Fan et al. 2009b). However, the metabolic rate of a 20 g mouse is much faster than a 70 kg human, as indicated by the approximately $3/4$ power scaling law of body mass (Downs et al. 2008; Glazier 2008; White et al. 2008) as well as the high heart (600 bpm) and respiration rate (200 breaths/min) of mice. Consequently, simply translating sampling times for human to mice based on differences in body mass is not warranted.

Here we report measurements of the time course of $^{13}\text{C}_6\text{-Glc}$ utilization following a bolus injection through the tail vein and determined the incorporation of ^{13}C labels into various metabolites extracted from different tissues and plasma to determine optimal sampling times for maximizing the extent of ^{13}C labeling. We also applied the methodologies to an orthotopic xenograft of human lung cancer cells in SCID mice to demonstrate the ability in revealing distinct ^{13}C labeling patterns of metabolites in the cancerous lung lobe.

2 Methods

2.1 Mouse handling

The murine tumor xenograft protocols were conducted in compliance with all guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Louisville. Fox Chase ICR SCID mice were purchased from Taconic (Hudson, NY) and maintained in a barrier facility at the University of Louisville according to institutional guidelines.

2.1.1 Orthotopic lung tumor xenograft—Human PC14PE6 cells were grown in RPMI medium as previously described (Fan et al. 2005), mixed with matrigel (total $100\ \mu\text{l}/10^6$ tumor cells) and injected into the left lung of ketamine-anesthetized mice. An equal volume of normal saline was injected into the right lung. Mice were fed ad libitum, and were monitored daily for signs of distress. Mice were sampled at 10 days post injection. Additional naïve SCID mice were used as controls (Onn et al. 2003).

2.2 $^{13}\text{C}_6\text{-glucose}$ infusion and plasma sampling

A 20% solution of $^{13}\text{C}_6\text{-Glc}$ in PBS was sterile-filtered and $100\ \mu\text{l}$ of this solution (i.e. 20 mg of $^{13}\text{C}_6\text{-Glc}$) were injected into the tail vein of a restrained mouse without anesthesia. Lower doses (e.g. 2 mg) were also examined for a few mice, which resulted in less ^{13}C labeling in tissue metabolites. Approximately $50\ \mu\text{l}$ of blood samples were taken intraorbitally at timed intervals, chilled on ice 5 min after standing at room temperature and separated into plasma and blood cells by centrifugation at 4°C at $3,500\times g$ for 15 min. Plasma was immediately flash-frozen in liquid N_2 for storage prior to metabolite extraction. The labeled glucose time course experiments were performed three times on separate dates, with two mice each for two of the experiments and five mice for the third experiment (see table below for lung cancer xenograft treatment and $^{13}\text{C}_6\text{-Glc}$ labeling time). Qualitatively

similar ^{13}C metabolite profiles of individual organs were obtained from these experiments at comparable time points, as estimated from 1-D ^1H - $\{^{13}\text{C}\}$ -HSQC spectra (cf. Figs. 6, S3 (in Supplementary material), and data not shown). Due to some variability of mice age, handling, and tumor status, it was impractical to average metabolite values from different individuals.

Exp #	# Mice/xenograft/ ^{13}C labeling time				
1	1/Yes/15 min	1/Yes/30 min			
2	1/Yes/15 min	2/No/30 min			
3	1/Yes/5 min	1/Yes/15 min	1/Yes/25 min	1/No/15 min	1/No/25 min

2.3 Tissue harvest

Mice were killed via decapitation at different times post $^{13}\text{C}_6$ -Glc injection, and the following organs were dissected sequentially: lung, heart, liver, kidney, brain, and thigh muscle. Dissected tissue was flash-frozen in liquid N_2 within 2–6 min of euthanasia.

2.4 Sample preparation

2.4.1 Plasma extraction—Twenty to thirty microliter of plasma was made to 10% trichloroacetic acid (TCA) concentration and centrifuged at 4°C , at $\geq 22,000\times g$ for 20 min to remove denatured proteins. The polar supernatant was lyophilized to remove TCA before preparation for NMR and GC–MS analysis.

2.4.2 Tissue extraction—Frozen tissues were ground in liquid N_2 to $<10\ \mu\text{m}$ particles in a 6750 Freezer/Mill (Retsch, Inc., Newtown, PA) and extracted simultaneously for soluble and lipidic metabolites as follows. Up to 20 mg of frozen tissue powder in 15 ml polypropylene conical centrifuge tube (Sarstedt, Newton, NC) containing three 3 mm diameter glass beads was vigorously shaken in 2 ml of cold acetonitrile (mass spectrometry grade, stored at -20°C) to denature proteins, followed by addition of 1.5 ml nanopure water, and 1 ml HPLC-grade chloroform (Fisher Scientific). The mixture was shaken vigorously until achieving a milky consistency (ca. 5 min), followed by centrifugation at $3,000\times g$ for 20 min at 4°C to separate the polar (top), lipidic (bottom), and tissue debris layers (interface). The polar and lipidic layers were recovered sequentially and the remaining tissue debris (mainly denatured proteins) was extracted again with 0.5 ml chloroform:methanol:butylated hydroxytoluene (BHT) (2:1:1 mM) and centrifuged at 4°C , $22,000\times g$ for 20 min to separate the three phases again. The residual polar and lipid fractions were pooled with respective main fractions. All three fractions were vacuum-dried in a speedvac device (Vacufuge, Eppendorf, New York, NY) and/or by lyophilization. The dry weight of tissue debris was obtained for normalization of metabolite content. The polar extracts were redissolved in 100% D_2O containing 30 nmol perdeuterated DSS (2,2'-dimethyl-2-silapentane-5-sulfonate, Cambridge Isotope Laboratories, Andover, MA) as internal chemical shift and concentration reference for NMR measurement.

2.4.3 NMR spectroscopy—NMR spectra were recorded at 14.1 T on a Varian Inova spectrometer equipped with a 5 mm inverse triple resonance cold probe, at 20°C . 1-D ^1H NMR spectra were recorded with an acquisition time of 2 s and a recycle time of 5 s to minimize peak saturation. 1-D ^1H Spectra were typically processed with zero filling to 131k points, and apodized with an unshifted Gaussian and a 0.5 Hz line broadening exponential. Concentrations of metabolites and ^{13}C incorporation were determined by peak integration of

the ^1H NMR spectra referenced to the intensity of DSS methyl groups, with correction for differential relaxation, as previously described (Fan and Lane 2008; Fan et al. 2008; Lane et al. 2008).

^{13}C profiling was achieved using 1-D ^1H - $\{^{13}\text{C}\}$ HSQC experiments recorded with a recycle time of 1.5 s and ^{13}C GARP decoupling during the proton acquisition time of 0.15 s. 1-D HSQC spectra were processed with zero-filling to 16k points and apodized using an unshifted Gaussian function and 6 Hz line broadening.

TOCSY and HSQC-TOCSY spectra were recorded with an isotropic mixing time of 50 ms, a B_1 field strength of 8 kHz, and acquisition times of 0.341 s in t_2 and 0.05 s in t_1 . The free induction decays were zero-filled once in t_2 , and linear predicted and zero filled to 4096 points in t_1 . The data were apodized using an unshifted Gaussian and a 1 Hz line broadening exponential in both dimensions. Positional ^{13}C incorporation into labeled metabolites was quantified as previously described (Lane and Fan 2007; Lane et al. 2008).

2.4.4 GC–MS analysis—GC–MS analysis was conducted on the same samples as used for NMR, as described previously (Fan et al. 2005). Briefly, following NMR analysis, an aliquot (50–100 μl) of the same extract was re-equilibrated with H_2O and lyophilized to remove deuterium, then silylated with 50 μl 1:1 (v/v) acetonitrile:MTBSTFA (*N*-methyl-*N*-[tert-butyldimethylsilyl]trifluoroacetamide) (Regis Chemical, Morton Grove, IL) by 3 h of sonication followed by standing overnight. The solution was directly injected into a PolarisQ GC-ion trap MSn (ThermoFinnigan, Austin, TX) with a 0.5 μl injection volume, using instrument conditions stated previously (Fan et al. 2005). Metabolites were identified and quantified automatically using Xcalibur software (ThermoFinnigan), based on their retention times and mass fragmentation patterns matched against an inhouse database and external standards. Identities were extensively verified by manual inspection. The %RSD for all GC–MS quantification was generally <3% in triplicate analyses for abundant metabolites (≥ 5 $\mu\text{mol/g}$ dry residue) such as lactate, and increased to 27% with metabolites ≤ 0.1 $\mu\text{mol/g}$ dry residue.

3 Results and discussion

3.1 Time course changes of plasma ^{13}C -glucose and ^{13}C -lactate

To assess the optimal time for transforming the tracer $^{13}\text{C}_6$ -glucose into downstream metabolites in mice, plasma samples were taken at 5 to 15-min intervals and processed for 1-D ^1H NMR analysis. Figure 1 shows a representative time course of the % ^{13}C enrichment in glucose (solid symbols) and lactate (open symbols) in the plasma of two SCID mice. The initial glucose enrichment ranged from 30 to 35% depending on the size of the mouse, suggesting that the plasma glucose concentration was increased by about 50% immediately after the bolus tracer injection. The % enrichment for glucose decreased rapidly and asymptotically within 1 h, presumably via the normal homeostatic mechanism.

Once taken up by tissues, labeled glucose was metabolized principally via glycolysis, which generated labeled lactate and to a lesser extent labeled alanine. Lactate is an important metabolite involved in pH and energy balance (Lane et al. 2009b). Cellular lactate was then exported into the blood and ultimately to the liver to be reconverted into glucose by gluconeogenesis (i.e. the Cori Cycle). ^{13}C -lactate in plasma therefore represents lactate newly synthesized since the ^{13}C glucose bolus, and is an indicator of systemic metabolism. This is consistent with the observed ^{13}C enrichment in lactate (Fig. 1), which started low, rapidly reached a maximum, and then depleted over longer periods, reflecting the decrease in the enrichment of the source glucose. The peak enrichment in lactate approached 12% during 15–20 min post injection in this experiment. A larger bolus in which the initial ^{13}C

glucose enrichment reached 65% was associated with a higher peak ^{13}C lactate level (data not shown). These data determined on average the optimal duration of tissue harvest for SIRM analysis to be 15–20 min post tracer infusion.

3.2 Tissue-dependent metabolism in six mouse tissues

3.2.1 GC–MS analysis—Individual tissues may absorb and metabolize glucose at different rates, which will be reflected in the time-dependent distribution of ^{13}C labeled metabolites in various tissues. Figure 2 shows the GC–MS analysis of ^{13}C -lactate isotopologue content of six different tissues dissected from three SCID mice, each obtained 5, 15, and 25 min after injection of the $^{13}\text{C}_6$ -glucose bolus. The $^{13}\text{C}_3$ -lactate (lactate+3) isotopologue was produced at the highest level in brain, followed by lung and kidney after only 5 min of glucose metabolism. Since $^{13}\text{C}_3$ -lactate is principally a product of glycolysis, it is expected that the highly glycolytic brain tissue would show the highest initial production of this isotopologue and maintenance of the initial level thereafter. In comparison, the $^{13}\text{C}_3$ -lactate level declined in lung and kidney while it peaked in heart and liver after 15 min of labeled glucose injection (Fig. 2). The time course of $^{13}\text{C}_3$ -lactate production in lung and kidney tracked closely with that of the plasma $^{13}\text{C}_6$ -glucose level (cf. Fig. 1), which could reflect a high rate of glucose oxidation coupled with a high rate of lactate consumption and/or export in these two organs (Bartlett et al. 1984; Longmore and Mourning 1976). Also notable is the delayed but very high production of $^{13}\text{C}_3$ -lactate in the heart, which could reflect its high-energy demand from contraction and preference for drawing energy from β -oxidation of fatty acids over glucose oxidation (Khairallah et al. 2004). Lactate has been shown to be both metabolized in the mitochondria and released by perfused mouse heart (Khairallah et al. 2004). Similar to the brain, muscle tissue maintained a constant but much lower production of $^{13}\text{C}_3$ -lactate from $^{13}\text{C}_6$ -glucose, which could be related to a significant contribution of unlabeled glycogen metabolism to lactate production in muscle (Woods and Krebs 1971).

Furthermore, readily detectable amounts of singly and doubly ^{13}C labeled lactate (lactate+1 and +2 or $^{13}\text{C}_1$ - and $^{13}\text{C}_2$ -lactate) was observed in all six tissues (Fig. 2). $^{13}\text{C}_1$ - and $^{13}\text{C}_2$ -lactate was present at comparable levels within 5 min of tracer injection and the change in their levels thereafter was small in brain, heart, and kidney. In contrast, $^{13}\text{C}_1$ -lactate level peaked in liver, lung and muscle after 15 min of tracer introduction and declined sharply thereafter. $^{13}\text{C}_2$ -lactate level also peaked after 15 min of metabolism in liver but changed little with time in muscle and lung. Neither $^{13}\text{C}_1$ -lactate nor $^{13}\text{C}_2$ -lactate can be produced from $^{13}\text{C}_6$ -glucose via glycolysis alone. Their production requires metabolic scrambling first through the non-oxidative branch of the pentose phosphate pathway (PPP) (Fig. 3a). Alternatively, in gluconeogenic tissues (i.e. liver and kidney), $^{13}\text{C}_1$ - and $^{13}\text{C}_2$ -lactate can also be produced from $^{13}\text{C}_6$ -glucose via the sequence of glycolysis, Krebs cycle, gluconeogenesis, and glycolysis again (Fig. 3b). Thus, the initial buildup of the scrambled lactate isotopologues in non-gluconeogenic tissues (brain, heart, lung, and muscle) could reflect the carbon flow through the PPP while the subsequent depletion in lung and muscle could be attributed to a combination of labeled glucose depletion in the plasma and lactate export or metabolism. The transient buildup of scrambled lactate in liver could result from PPP and gluconeogenic activity, in part using labeled lactate imported from the blood (Cori cycle).

In addition to lactate, ^{13}C -labeled isotopologue series for a number of metabolites were quantified by GC–MS analysis. The time course changes in Ala, succinate, Asp, Glu, Gln are shown in Fig. 4, while those for fumarate, malate, citrate, GAB, Ser, Asn, and Gly are illustrated in Fig. S2 (Supplementary material). The time course of ^{13}C labeling for Ala was generally similar to that of lactate for all tissues but lung, muscle, and kidney. Most notably,

the level of the three ^{13}C isotopologues of Ala in lung peaked at 15 min, in contrast to the $^{13}\text{C}_3$ -lactate level in lung which began to decline 5 min after $^{13}\text{C}_6$ -glucose injection. Both labeled Ala and lactate share the same precursor, i.e. labeled pyruvate derived from glycolysis and/or PPP. Yet they differed in their time course behavior in the lung and muscle. This implied the presence of two separate pools of pyruvate in the two tissues each leading to lactate and Ala synthesis. A similar observation was made for human lung cancer tissues (Fan et al. 2009b). As described for lactate, the scrambled $^{13}\text{C}_1$ - and $^{13}\text{C}_2$ -Ala reflected transformations of $^{13}\text{C}_6$ -glucose via PPP and/or gluconeogenesis.

The ^{13}C -isotopologue series of succinate, Asp, Glu, and Gln were clearly present. Some of these reached relatively high levels, e.g. $^{13}\text{C}_1/^{13}\text{C}_2$ -Asp in brain, $^{13}\text{C}_1$ -Asp in lung, $^{13}\text{C}_1/^{13}\text{C}_2/^{13}\text{C}_3$ -Glu in brain, $^{13}\text{C}_1/^{13}\text{C}_2$ -Glu in kidney, $^{13}\text{C}_1$ -Glu in lung, $^{13}\text{C}_1/^{13}\text{C}_2$ -Glu in liver, $^{13}\text{C}_1/^{13}\text{C}_2$ -Glu in heart, $^{13}\text{C}_1/^{13}\text{C}_2$ -Gln in brain heart, and liver, $^{13}\text{C}_1/^{13}\text{C}_2/^{13}\text{C}_3/^{13}\text{C}_4$ -succinate in liver, as well as $^{13}\text{C}_1/^{13}\text{C}_2$ -succinate in kidney, brain, and heart. $^{13}\text{C}_2$ -succinate, $^{13}\text{C}_2$ -Asp, $^{13}\text{C}_2$ -Glu, and $^{13}\text{C}_2$ -Gln can be derived from $^{13}\text{C}_6$ -glucose via glycolysis plus the 1st turn of the Krebs cycle (cf. Fig. 3b). These doubly labeled isotopologues along with the $^{13}\text{C}_1$ -isotopologues could also be produced from a second turn of the Krebs cycle according to the scheme in Fig. 3c, where the starting $^{13}\text{C}_2$ -oxaloacetate (OAA) is derived from $^{13}\text{C}_6$ -Glc but acetyl CoA is produced from unlabeled glucose. Based on the dominance of the $^{13}\text{C}_1$ -isotopologues of Asp, Glu, and Gln (branched metabolites of the Krebs cycle) in most tissues, a major fraction of the labeled glucose had undergone at least two turns of the cycle. In fact, some $^{13}\text{C}_6$ -Glc may have passed through a third turn, as evidenced by the production of $^{13}\text{C}_1$ -citrate (Fig. S2, Supplementary material), following the same scheme as in Fig. 3c. The unequal synthesis of $^{13}\text{C}_1$ and $^{13}\text{C}_2$ isotopologues of Asp, Glu, and Gln in most cases suggests additional source(s) of labeled carbon input into the Krebs cycle, e.g. $^{13}\text{C}_1$ -acetyl CoA (derived from scrambled labeled lactate in Fig. 3a, b) plus unlabeled OAA will lead to the production of $^{13}\text{C}_1$ -Glu, -Gln, and -Asp only.

The unusual abundance of $^{13}\text{C}_1/^{13}\text{C}_2$ -Asp, $^{13}\text{C}_1/^{13}\text{C}_2$ -Glu, and $^{13}\text{C}_1/^{13}\text{C}_2$ -Gln in the brain suggests a high flux through the Krebs cycle for the production of neurotransmitters (Glu, Gln in Fig. 4; GAB in Fig. S2, Supplementary material). GAB is produced from Glu via Glu decarboxylase activity. There was also an appreciable presence of $^{13}\text{C}_3/^{13}\text{C}_4$ -Asp, $^{13}\text{C}_3/^{13}\text{C}_4$ -Glu, and $^{13}\text{C}_3/^{13}\text{C}_4$ -succinate in all six tissues (Fig. 4), which could be produced via the second and third turn of the Krebs cycle with input of both labeled acetyl CoA and OAA (cf. Fan et al. 2010), as opposed to Fig. 3c where only starting OAA is ^{13}C -labeled. However, $^{13}\text{C}_3$ -Asp could also be derived from the carboxylation of $^{13}\text{C}_3$ -pyruvate (PC) via the anaplerotic pyruvate carboxylase activity (Fan et al. 2009b,2010). PC coupled with the first turn of the Krebs cycle would lead to the production of $^{13}\text{C}_3$ -citrate while $^{13}\text{C}_4$ - and $^{13}\text{C}_5$ -citrate would be the expected products from the second and third turn of the Krebs cycle activity, respectively (Fan et al. 2010). $^{13}\text{C}_3$ -citrate was largely present at a higher level than those of $^{13}\text{C}_4$ - and $^{13}\text{C}_5$ -citrate in all six tissues (Fig. S2, Supplementary material), which suggests that PC contributed substantially to the production of $^{13}\text{C}_3$ -Asp in these tissues. The relatively high PC activity in the brain, heart, and liver, where PC is strongly expressed (Jitrapakdee et al. 2008) is supported by the high level of $^{13}\text{C}_3$ -Asp (Fig. 4) and abundance of $^{13}\text{C}_2$ -Glu and $^{13}\text{C}_3$ -Glu/Gln (cf. Fig. 6), which can be derived from $^{13}\text{C}_6$ -glucose via glycolysis, PC, and the first turn of the Krebs cycle (Fan et al. 2010).

3.2.2 NMR analysis—The same set of extracts shown in Fig. 2 were analyzed by NMR to complement and verify the GC–MS analysis. The ^{13}C enrichment in glucose/glucose-6-phosphate (not resolved) and lactate was determined by 1-D ^1H NMR. The enrichments from 5 to 25 min post infusion were substantial in all tissues, except for glucose and lactate in kidney where ^{13}C enrichment was not determined after 25 min of metabolism due to

interferences (Table 1). The peak % lactate enrichments differed presumably according to the metabolic activity and/or glucose uptake rates of the six tissues measured (Table 1).

More extensive ^{13}C labeling patterns in metabolites was determined by two-dimensional NMR experiments. Figure 5 shows a high-resolution 2-D ^1H - ^{13}C HSQC-TOCSY (heteronuclear single quantum coherence-total correlation spectroscopy) spectrum (Fig. 5b) of a lung extract obtained from a SCID mouse 15 min after injecting $^{13}\text{C}_6$ -glucose. The spectrum was acquired with sufficient digital resolution to resolve ^{13}C coupling patterns of labeled resonances, which can be more clearly visualized in the 1-D projection spectrum onto the ^{13}C dimension (Fig. 5a). The HSQC-TOCSY data not only confirmed the identity of ^{13}C -labeled metabolites from their characteristic ^1H - ^{13}C and ^1H - ^1H covalent linkages but also enabled determination of the labeled carbon position, i.e. positional isotopomers (Fan and Lane 2008). For example, lactate was confirmed by the covalent linkages represented by cross-peaks from H-3 to C-3, H-2 to C-2, H-3 to H-2, and H-2 to H-3 of lactate (traced by red rectangle in Fig. 5b). The ^{13}C -coupling pattern of C-3 and C-2 of lactate was respectively doublet and triplet (Fig. 5a), indicating that lactate was largely labeled at all three carbon positions. This is consistent with the abundance of $^{13}\text{C}_3$ -lactate by GC-MS analysis of the same extract (cf. Fig. 2). Similarly, the doublet pattern of C-1 and C-6 and triplet pattern of C-2, C-3, and C-4 of glucose-6-phosphate (Glc-6-P) indicate the presence of $^{13}\text{C}_6$ -Glc-6-P in the extract, which was derived from $^{13}\text{C}_6$ -glucose via hexose kinase or glucokinase activity. This information and the labeled patterns of glutathione, adenine nucleotides, and UDP-sugars were not obtainable by GC-MS. Moreover, the N-methyl carbons of phosphocholine and C-2 of Gly were singlets, which suggests that they were largely contributed from the natural abundance ^{13}C , and therefore not derived from $^{13}\text{C}_6$ -glucose.

Once the labeled metabolites were assigned by 2-D HSQC methods, the 1-D HSQC analysis of ^1H directly attached to ^{13}C provided a semi-quantitative overview of the abundance of various ^{13}C -labeled metabolites in the six tissues (Fig. 6). It is clear that different tissues exhibited rather distinct patterns of labeled glucose metabolism, as reflected by the different 1-D HSQC profiles. Brain tissue built up a considerable pool of ^{13}C -3-Asp, ^{13}C -2, 3, or 4-Glu/Gln and ^{13}C -2, 3, or 4- γ -aminobutyrate (GAB), which is consistent with its high Krebs cycle activity and specialized production of neurotransmitters Glu, Gln, and GAB, as evident also from the GC-MS data (Fig. 4). There was no detectable free ^{13}C -labeled glucose in the brain tissue, which could be related to its high glycolytic activity (cf. Fig. 2) and high requirement for glucose in energy production (Owen et al. 1967). Heart tissue was also high in labeled Glu, Gln and succinate, which reflects a high Krebs cycle activity. It also had the highest labeled lactate level among the six tissues and an appreciable level of labeled Glc-6-P, which suggests a high glucose uptake and glycolytic rates. Liver tissue had the highest Glc-6-P but a moderate level of labeled lactate, which could be a result of fast glucose uptake and gluconeogenesis from lactate. Liver was the only tissue where labeled glycogen was observed, which could be attributed to its high capacity of glycogen synthesis (Hers 1976). Muscle tissue had the second highest labeled lactate level, which is consistent with a high rate of glycolysis. In addition, it was the only tissue where the ribose moiety of adenine nucleotides (AXP) was labeled, which suggests that muscle possesses high PPP activity. This is consistent with the high levels of scrambled labeled lactate in the muscle tissue as revealed by GC-MS analysis (cf. Fig. 2). Lung and kidney tissues had the lowest overall labeled metabolite content, which suggests a lower rate of glucose metabolism.

It should be noted that the above-described ^{13}C -labeling patterns of metabolites were acquired from SCID mice after a brief period (<5 min) of restraint for ^{13}C -glucose injection. Prolonged animal handling is known to cause tachycardia and release of stress hormones into the plasma of rodents (Kawashima et al. 1985; Roizen et al. 1978), which could in turn

affect metabolic activity of various organs, such as glycogen degradation in liver (Gruetter et al. 1994), synaptic release of amino acids in brain (Timmerman et al. 1999), and energy metabolism in brain (Chance et al. 1978). Therefore, handling stress-induced alteration of ^{13}C -glucose metabolism may contribute to the pattern of observed labeled products in Figs. 2, 4, 5, 6, 7, 8. Particularly notable is the lack of ^{13}C -glucose and the considerable buildup of ^{13}C -lactate in our mouse brain extracts (cf. Figs. 6, S3 (Supplementary material)), which is distinct from that observed in situ in human or rat brain (Gruetter et al. 2003). Nevertheless, it is reasonable to conclude that the glucose-dependent metabolic activities differed among the various tissue types of mice.

3.3 Metabolism in normal versus tumorous lung tissues

$^{13}\text{C}_6$ -glucose was used as a tracer to track changes in metabolic pathways associated with lung tumor development in SCID mice. Figure 7 compares 1-D HSQC NMR spectra of a tumorous lung with its paired normal lung dissected from the same mouse 15 min after the tracer infusion. It is clear that the ^{13}C abundance of various positional isotopomers of metabolite (as reflected by the resonance intensity of their protons attached to ^{13}C) were higher in tumorous lung than its normal counterpart. These included ^{13}C -2 and 3-lactate, ^{13}C -3-Ala, ^{13}C -3 and 4-Pro, ^{13}C -4-Glu, ^{13}C -4-Gln, ^{13}C -4-glutamyl moiety of GSH (^{13}C -4-Glu-GSH), ^{13}C -2,3-succinate, ^{13}C -2-Gly, ^{13}C -1 to 6-glucose (Glc), and N-methyl-phosphocholine (NMe-PCholine). The NMe-PCholine signal was attributed to natural abundance of ^{13}C since the choline moiety is not expected to be synthesized from $^{13}\text{C}_6$ -glucose.

A separate set of tumorous and normal lung tissue extracts similarly prepared as in Fig. 7 were quantified by GC-MS, as shown in Figs. 8 and S4 in the Supplementary material. The GC-MS quantification agreed with the NMR analysis in terms of the greater production of ^{13}C labeled lactate, Ala, succinate, fumarate, Asp, Glu, Gly, and Ser in the tumorous than in normal lung tissues. Some of these metabolites, i.e. ^{13}C -labeled malate, fumarate, and Ser were obscured by others in the 1-D HSQC spectra in Fig. 7 but were clearly quantified by GC-MS. Moreover, as described above, increased carbon flow through specific pathways in the tumorous lung could be deduced based on the combined ^{13}C mass isotopologue series of metabolites acquired from GC-MS and the ^{13}C positional isotopomer information obtained from the NMR analysis. For example, enhanced buildup of ^{13}C -glucose in the tumorous lung (Fig. 7) may be related to increased glucose uptake. The increased production of $^{13}\text{C}_3$ -lactate and -Ala is consistent with enhanced glycolysis while the larger buildup of $^{13}\text{C}_1$ and $^{13}\text{C}_2$ -lactate and -Ala suggests more active PPP in the tumorous lung. Enhanced Krebs cycle activity was evident from the greater accumulation of $^{13}\text{C}_1$ - and $^{13}\text{C}_2$ -malate, -succinate, -fumarate, and -Glu in the tumorous lung (cf. Fig. 3b, c schemes). Activation of PC in the tumorous lung tissue could be inferred from the increased buildup of $^{13}\text{C}_3$ -Asp (Fig. 8) and the presence of $^{13}\text{C}_3$ -citrate isotopologue (data not shown), which is a unique product of PC. The higher synthesis of $^{13}\text{C}_2$ -Gly in the tumorous lung suggests a more active one-carbon metabolism associated with tumor development.

The buildup of ^{13}C -labeled glucose and lactate was previously observed by in vivo NMR in RIF-1 tumor xenograft in mice under relatively fast and slow infusion of ^{13}C -1-Glc and anesthesia (Artemov et al. 1995, 1998). However, it was concluded in the RIF-1 tumor study (Artemov et al. 1998) that no intracellular glucose or glycolytic products were observable in vivo, which implies that the ^{13}C -glucose and lactate NMR signals were originated from the blood. In the case of lung tumors resected from human patients (Fan et al. 2009a, b), glucose levels were also very low in tumors with poor vascularization. Therefore, it is possible that the accumulation of glucose in the lung tumor xenograft in the present study may have appreciable contribution from the blood. The same situation can be said for lactate accumulation. However, we note that the fractional ^{13}C enrichment of lactate in the blood

was considerably lower than that in the tissue (cf. Table 1, Fig. 1) from the same animals, suggesting that a substantial fraction of the observed metabolism was from the target tissue. As in vivo lactate level can increase significantly by reducing blood flow to the tumor (Bhujwala et al. 1994), additional lactate buildup in our excised lung tumor could also be attributed to the time it took to dissect (i.e. 2–6 min) before liquid N₂ freezing. However, in contrast to the previous in vivo analysis of RIF-1 tumors (Artemov et al. 1998), the excised tumorous lung in this study contained an appreciable amount of ¹³C labeled Krebs cycle intermediates and Ala (a glycolytic product), suggesting a more active Krebs cycle and glycolytic activity. This distinction may be attributed to a much smaller (1–2 mm) and thus earlier stage of tumor, different tumor type (orthotopic adenocarcinoma instead of ectopic fibrosarcoma), mouse strain (SCID instead of C₃H/HeN), and/or animal handling (restraint during labeled glucose administration instead of anesthesia) in the present study. As the tumor burden was small, the true metabolic differences between the tumor-bearing lung and the paired nontumorous lung are presumably underestimated.

In conclusion, differential rates of glucose transformations through various metabolic pathways in six different tissues plus blood plasma from SCID mice can be discerned with tail-vein injection of a bolus of ¹³C₆-Glc. The ¹³C labeled patterns of metabolites in terms of isotopologues and isotopomers obtained from GC–MS and NMR analyses enabled reconstruction of multiple pathways including glycolysis, pentose phosphate pathway, multiple turns of the Krebs cycle, anaplerotic pyruvate carboxylation, Gly biosynthesis, nucleotide biosynthesis, and gluconeogenesis. When applied to human lung cancer cell xenograft in SCID mice, enhanced glucose uptake, glycolysis, PPP, Krebs cycle, pyruvate carboxylation, and Gly biosynthesis were found to be associated with tumor development, which is analogous to metabolic distinctions observed in human lung cancer tissues (Fan et al. 2009b).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

BHT	Butylated hydroxytoluene
DSS	2,2'Dimethylsilapentane-5-sulfonate
MTBSTFA	<i>N</i> -methyl- <i>N</i> -[tert-butyl(dimethylsilyl)]trifluoroacetamide
NSCLC	Non small cell lung cancer
SIRM	Stable isotope resolved metabolomics

Acknowledgments

This work was supported in part by National Science Foundation EPSCoR grant # EPS-0447479, NIH Grant Number P20RR018733 from the National Center for Research Resources, 1R01CA118434-01A2 (to TWMF), R01CA-086412 and RO1 CA150947 (to JY) from the National Cancer Institute, the Kentucky Challenge for Excellence, Susan G. Komen Foundation BCTR0503648 and NCI R21CA133668 (to ANL), and the Brown Foundation. We thank J. Tan, Ruifeng Su and Mr. Richard Hansen for technical assistance. We also thank the two reviewers for many useful comments and additional references to improve the manuscript.

References

- Artemov D, Bhujwala ZM, Glickson JD. In vivo selective measurement of (1–¹³C)-glucose metabolism in tumors by heteronuclear cross polarization. *Magnetic Resonance in Medicine*. 1995; 33:151–155. [PubMed: 7707903]

- Artemov D, Bhujwala ZM, Pilatus U, Glickson JD. Two-compartment model for determination of glycolytic rates of solid tumors by in vivo ¹³C NMR spectroscopy. *NMR in Biomedicine*. 1998; 11:395–404. [PubMed: 10221582]
- Bak LK, Waagepetersen HS, Melo TM, Schousboe A, Sonnewald U. Complex glutamate labeling from [U-C-13]glucose or [U-C-13]lactate in co-cultures of cerebellar neurons and astrocytes. *Neurochemical Research*. 2007; 32:671–680. [PubMed: 17021949]
- Bartlett S, Espinal J, Janssens P, Ross BD. The influence of renal function on lactate and glucose metabolism. *Biochemical Journal*. 1984; 219:73–78. [PubMed: 6721865]
- Beger R, Hansen D, Schnackenberg L, Cross B, Fatollahi J, Lagunero F, et al. Single valproic acid treatment inhibits glycogen and RNA ribose turnover while disrupting glucose-derived cholesterol synthesis in liver as revealed by the [U-13C6]-d-glucose tracer in mice. *Metabolomics*. 2009; 5:336–345. [PubMed: 19718458]
- Bhujwala ZM, Shungu DC, Chatham JC, Wehrle JP, Glickson JD. Glucose metabolism in RIF-1 tumors after reduction in blood flow: an in vivo ¹³C and ³¹P NMR study. *Magnetic Resonance in Medicine*. 1994; 32:303–309. [PubMed: 7984062]
- Chance B, Nakase Y, Bond M, Leigh JS Jr, McDonald G. Detection of ³¹P nuclear magnetic resonance signals in brain by in vivo and freeze-trapped assays. *Proceedings of the National Academy of Sciences of the United States of America*. 1978; 75:4925–4929. [PubMed: 283403]
- DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, et al. Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:19345–19350. [PubMed: 18032601]
- Delgado TC, Castro MM, Geraldles CF, Jones JG. Quantitation of erythrocyte pentose pathway flux with [2-(13)]glucose and H-1 NMR analysis of the lactate methyl signal. *Magnetic Resonance in Medicine*. 2004; 51:1283–1286. [PubMed: 15170851]
- Downs CJ, Hayes JP, Tracy CR. Scaling metabolic rate with body mass and inverse body temperature: A test of the Arrhenius fractal supply model. *Functional Ecology*. 2008; 22:239–244.
- Fan T, Bandura L, Higashi R, Lane A. Metabolomics-edited transcriptomics analysis of Se anticancer action in human lung cancer cells. *Metabolomics Journal*. 2005; 1:325–339.
- Fan TWM, Higashi RM, Lane AN. An in vivo H-1 and P-31 NMR investigation of the effect of nitrate on hypoxic metabolism in maize roots. *Archives of Biochemistry and Biophysics*. 1988; 266:592–606. [PubMed: 3190244]
- Fan TWM, Higashi RM, Lane AN, Jardetzky O. Combined use of proton NMR and gas chromatography-mass spectra for metabolite monitoring and in vivo proton NMR assignments. *Biochimica et Biophysica Acta*. 1986; 882:154–167. [PubMed: 3011112]
- Fan TW-M, Kucia M, Jankowski K, Higashi RM, Rataczjak MZ, Rataczjak J, et al. Proliferating Rhabdomyosarcoma cells shows an energy producing anabolic metabolic phenotype compared with primary myocytes. *Molecular Cancer*. 2008; 7:79. [PubMed: 18939998]
- Fan TW, Lane AN. Structure-based profiling of metabolites and isotopomers by NMR. *Progress in NMR Spectroscopy*. 2008; 52:69–117.
- Fan TWM, Lane AN, Higashi RM. In vivo and in vitro metabolomic analysis of anaerobic rice coleoptiles revealed unexpected pathways. *Russian Journal of Plant Physiology*. 2003; 50:787–793.
- Fan TW, Lane AN, Higashi RM, Bousamra M II, Kloecker G, Miller DM. Metabolic profiling identifies lung tumor responsiveness to erlotinib. *Experimental and Molecular Pathology*. 2009a; 87:83–86. [PubMed: 19409891]
- Fan TW, Lane AN, Higashi RM, Farag MA, Gao H, Bousamra M, et al. Altered regulation of metabolic pathways in human lung cancer discerned by ¹³C stable isotope-resolved metabolomics (SIRM). *Molecular Cancer*. 2009b; 8:41. [PubMed: 19558692]
- Fan TW-M, Yuan P, Lane AN, Higashi RM, Wang Y, Hamidi A, et al. Stable isotope-resolved metabolomic analysis of lithium effects on glial-neuronal metabolism and interactions. *Metabolomics*. 2010; 6:165–179. [PubMed: 20631920]
- Glazier DS. Effects of metabolic level on the body size scaling of metabolic rate in birds and mammals. *Proceedings of the Royal Society B: Biological Sciences*. 2008; 275:1405–1410.

- Gruetter R, Adriany G, Choi IY, Henry PG, Lei H, Oz G. Localized in vivo ^{13}C NMR spectroscopy of the brain. *NMR in Biomedicine*. 2003; 16:313–338. [PubMed: 14679498]
- Gruetter R, Magnusson I, Rothman DL, Avison MJ, Shulman RG, Shulman GI. Validation of ^{13}C NMR measurements of liver glycogen in vivo. *Magnetic Resonance in Medicine*. 1994; 31:583–588. [PubMed: 8057810]
- Hers HG. The control of glycogen metabolism in the liver. *Annual Review of Biochemistry*. 1976; 45:167–189.
- Jitrapakdee S, St Maurice M, Rayment I, Cleland WW, Wallace JC, Attwood PV. Structure, mechanism and regulation of pyruvate carboxylase. *Biochemical Journal*. 2008; 413:369–387. [PubMed: 18613815]
- Kawashima K, Miwa Y, Fujimoto K, Matsumoto J, Kimura M, Nagakura A. Hypotensive effect of bunitrolol at low plasma concentrations in conscious, unrestrained spontaneously hypertensive rats. *Japanese Journal of Pharmacology*. 1985; 38:259–265. [PubMed: 2865388]
- Khairallah M, Labarthe F, Bouchard B, Danialou GT, Petrof BJ, Des Rosiers C. Profiling substrate fluxes in the isolated working mouse heart using C-13-labeled substrates: Focusing on the origin and fate of pyruvate and citrate carbons. *American Journal of Physiology: Heart and Circulatory Physiology*. 2004; 286:H1461–H1470. [PubMed: 14670819]
- Lane AN, Fan TW. Quantification and identification of isotopomer distributions of metabolites in crude cell extracts using ^1H TOCSY. *Metabolomics*. 2007; 3:79–86.
- Lane AN, Fan TW, Higashi RM. Isotopomer-based metabolomic analysis by NMR and mass spectrometry. *Methods in Cell Biology*. 2008; 84:541–588. [PubMed: 17964943]
- Lane AN, Fan TW-M, Higashi RM. Metabolic acidosis and the importance of balanced equations. *Metabolomics*. 2009a; 5:163–165.
- Lane AN, Fan TW, Higashi RM, Tan J, Bousamra M, Miller DM. Prospects for clinical cancer metabolomics using stable isotope tracers. *Journal of Experimental and Molecular Pathology*. 2009b; 86:165–173.
- Longmore WJ, Mourning JT. Lactate production in isolated perfused rat lung. *American Journal of Physiology*. 1976; 231:351–354. [PubMed: 961886]
- Mancuso A, Zhu AZ, Beardsley NJ, Glickson JD, Wehrli S, Pickup S. Artificial tumor model suitable for monitoring P-31 and C-13 NMR spectroscopic changes during chemotherapy-induced apoptosis in human glioma cells. *Magnetic Resonance in Medicine*. 2005; 54:67–78. [PubMed: 15968647]
- Mason GF, Petersen KF, de Graaf RA, Shulman GI, Rothman DL. Measurements of the anaplerotic rate in the human cerebral cortex using C-13 magnetic resonance spectroscopy and [1-C-13] and [2-C-13] glucose. *Journal of Neurochemistry*. 2007; 100:73–86. [PubMed: 17076763]
- Mendes AC, Caldeira MM, Silva C, Burgess SC, Merritt ME, Gomes F, et al. Hepatic UDP-glucose C-13 isotopomers from [U-C-13]glucose: A simple analysis by C-13 NMR of urinary menthol glucuronide. *Magnetic Resonance in Medicine*. 2006; 56:1121–1125. [PubMed: 17036288]
- Onn A, Isobe T, Itasaka S, Wu WJ, O'Reilly MS, Hong WK, et al. Development of an orthotopic model to study the biology and therapy of primary human lung cancer in nude mice. *Clinical Cancer Research*. 2003; 9:5532–5539. [PubMed: 14654533]
- Owen OE, Morgan AP, Kemp HG, Sullivan JM, Herrera MG, Cahill GF Jr. Brain metabolism during fasting. *Journal of Clinical Investigation*. 1967; 46:1589–1595. [PubMed: 6061736]
- PaineMurrieta GD, Taylor CW, Curtis RA, Lopez MHA, Dorr RT, Johnson CS, et al. Human tumor models in the severe combined immune deficient (SCID) mouse. *Cancer Chemotherapy and Pharmacology*. 1997; 40:209–214. [PubMed: 9219503]
- Richmond A, Su YJ. Mouse xenograft models vs GEM models for human cancer therapeutics. *Disease Models & Mechanisms*. 2008; 1:78–82. [PubMed: 19048064]
- Roizen MF, Moss J, Henry DP, Weise V, Kopin IJ. Effect of general anesthetics on handling- and decapitation-induced increases in sympathoadrenal discharge. *Journal of Pharmacology and Experimental Therapeutics*. 1978; 204:11–18. [PubMed: 563442]
- Thornburg JM, Nelson KK, Lane AN, Arumugam S, Simmons A, Eaton JW, et al. Targeting aspartate aminotransferase in breast cancer. *Breast Cancer Research*. 2008; 10:R84. [PubMed: 18922152]

- Timmerman W, Cisci G, Nap A, de Vries JB, Westerink BH. Effects of handling on extracellular levels of glutamate and other amino acids in various areas of the brain measured by microdialysis. *Brain Research*. 1999; 833:150–160. [PubMed: 10375690]
- Vizan P, Boros LG, Figueras A, Capella G, Manges R, Bassilian S, et al. K-ras codon-specific mutations produce distinctive metabolic phenotypes in human fibroblasts. *Cancer Research*. 2005; 65:5512–5515. [PubMed: 15994921]
- White CR, Terblanche JS, Kabat AP, Blackburn TM, Chown SL, Butler PJ. Allometric scaling of maximum metabolic rate: The influence of temperature. *Functional Ecology*. 2008; 22:616–623.
- Woods HF, Krebs HA. Lactate production in the perfused rat liver. *Biochemical Journal*. 1971; 125:129–139. [PubMed: 5158899]
- Zwingmann C, Leibfritz D. Regulation of glial metabolism studied by C-13-NMR. *NMR in Biomedicine*. 2003; 16:370–399. [PubMed: 14679501]

Fig. 1.

Glucose consumption and lactate production in two SCID mice. Plasma processing and metabolite analysis by 1-D ^1H NMR were described in "Methods. % ^{13}C enrichment was calculated from the intensity of ^{13}C satellites and central peaks of H-3 of lactate as described in Lane et al. (2008). Filled circle, filled square: Glucose; open circle, open square: Lactate

Fig. 2.

Representative GC-MS analysis of ^{13}C -isotopologue series of lactate in six different SCID mouse tissues. The six tissues were obtained from three SCID mice, injected with $^{13}\text{C}_6\text{-Glc}$ and dissected 5, 15, and 25 min thereafter. Their polar extracts were prepared and analyzed by GC-MS as described in "Methods". The values displayed are the ^{13}C enrichment values for the tissue extracts in $\mu\text{mole/g dry residue weight}$. The dry residue weight was obtained from tissue residues remained after polar and lipid extractions. Lactate+1 to +3 are singly, doubly, and triply ^{13}C -labeled isotopologues of lactate

Fig. 3. Tracking of ^{13}C atoms from $^{13}\text{C}_6$ -glucose to ^{13}C -lactate through glycolysis, pentose phosphate pathway, Krebs cycle, and gluconeogenesis. **a** The ^{13}C flow through the non-oxidative branch of PPP and glycolysis in the presence of $^{13}\text{C}_6$ -glucose + unlabeled glucose, **b** tracks ^{13}C atoms from glycolysis, Krebs cycle, gluconeogenesis, and glycolysis again, and **c** traces ^{13}C flow in the 2nd turn of the Krebs cycle, where ^{13}C -OAA is derived from (b) after the 1st turn and acetyl CoA is generated from unlabeled glucose via glycolysis. Black dots represent unlabeled carbons while color dots denote ^{13}C . The pink and green dots in (b) after the SCS step illustrates scrambled ^{13}C . Dashed black arrows denote multiple reactions steps, double-headed black arrows indicate reversible reactions, and dashed green arrows denote site of carbon-carbon bond break. PDH pyruvate dehydrogenase, SCS succinyl CoA synthetase, PEPCK phosphoenolpyruvate carboxykinase, OAA oxaloacetate, α -KG α -ketoglutarate, PEP phosphoenolpyruvate. (Color figure online)

Fig. 4. Representative GC–MS analysis of ^{13}C -isotopologue series of selected metabolites in different SCID mouse tissues. The same polar extracts as in Fig. 2 were analyzed by GC–MS and quantified for ^{13}C isotopologues for various metabolites. The values displayed are the ^{13}C enrichment values for the tissue extracts in $\mu\text{mol/g}$ dry residue weight, as in Fig. 2. Metabolite+1 to 5 are singly, doubly, triply, quadruply, and quintuply ^{13}C -labeled isotopologues

Fig. 5. 2-D ^1H - ^{13}C HSQC-TOCSY analysis of SCID mouse lung extracts. $^{13}\text{C}_6$ -glucose was infused into SCID mouse via tail vein and lung tissue was dissected, pulverized, and extracted as described in “Methods”. The 2-D spectrum, displayed as a contour plot (**b**), was acquired at 14.1 T, processed with linear prediction in the ^{13}C dimension and zero-filling to $4\text{k} \times 2\text{k}$ real digital points. Also displayed is the 1-D projection spectrum along the ^{13}C dimension (**a**). The acquisition time in t_1 (50 ms) was sufficient to resolve $^1J_{\text{CC}}$ scalar couplings (40–50 Hz) in the ^{13}C dimension. *Rectangular boxes* trace some of the covalent linkages represented by the 2-D cross-peaks. *GSH* reduced glutathione, *Glc* glucose, *PCr-NMe*, *PC-NMe* N-methyl carbon of phosphocreatine or phosphocholine, respectively, *AXP* adenine nucleotides

Fig. 6. 1-D HSQC spectral comparison of six SCID mouse tissue extracts labeled with $^{13}\text{C}_6$ -glucose. SCID mice were infused with $^{13}\text{C}_6$ -glucose for 15 min before tissue dissection, extraction, and analysis by NMR as described in “Methods”. The 1-D HSQC spectra of all six tissues were normalized to tissue residue weight (remained after polar and lipid extractions) and spectral parameters so that the intensity or metabolite resonances reflected their tissue content

Fig. 7.

1-D HSQC spectral comparison of tumorous versus paired normal lung. A SCID mouse was injected with 1 million PC14PE6 lung cancer cells in matrigel in one of the lung lobes while the other lung lobe received saline only. After lung tumor establishment, the animal received $^{13}\text{C}_6$ -glucose (Glc) 15 min prior to tissue dissection, extraction, and NMR analysis as described in "Methods". The 1-D HSQC spectra were normalized to tissue residue weight (remained after polar and lipid extractions) and spectral parameters so that the intensity or metabolite resonances reflected their tissue content. *NMe-PCholine* *N*-methyl resonance of phosphocholine

Fig. 8.

Representative GC-MS quantification of ^{13}C -isotopologue series of metabolites in tumorous and normal lung tissues of SCID mice. Tumorous and normal lung tissues were obtained from two SCID mice, similarly treated as in Fig. 7. Their polar extracts were analyzed by GC-MS as described in "Methods". The values displayed are the difference in ^{13}C enrichment between tumorous and normal lung tissue extracts in $\mu\text{mol/g}$ dry residue weight. The dry residue weight was obtained from tissue remained after polar and lipid extractions. Metabolite+1 to 5 are singly, doubly, triply, quadruply, and quintuply ^{13}C -labeled isotopologues

Table 1% ^{13}C enrichment in tissue lactate and glucose

Tissue	Time (min)	% ^{13}C lactate	% ^{13}C glucose
Brain	5	47	81
	15	47	45
	25	47	54
Heart	5	25	48
	15	44	23
	25	47	36
Kidney	5	36	59
	15	18	38
	25	ND	ND
Liver	5	39	20
	15	40	8
	25	53	29
Lung	5	27	23
	15	28	29
	25	39	32
Muscle	5	33	47
	15	50	24
	25	61	37

% ^{13}C in lactate was calculated from the intensity of ^{13}C satellites and central peak of H-3, while % ^{13}C in glucose was similarly calculated based on the intensity of ^{13}C satellites and central peak of H-1 α measured from 1-D ^1H NMR spectra, as described in Lane et al. (2008). *ND* not determined. Estimated errors were ± 2 to 5%