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Novel diagnostics of metabolic dysfunction detected in breath and plasma by selective isotope assisted labeling (SIAL)

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Abstract

OBJECTIVE—Metabolomics is the study of a unique fingerprint of small molecules present in biological systems under healthy and disease conditions. One of the major challenges in metabolomics is validation of fingerprint molecules to identify specifically perturbed pathways in metabolic aberrations. This step is crucial to the understanding of budding metabolic pathologies and the ability to identify early indicators of common diseases such as obesity, diabetes mellitus type II, metabolic syndrome, polycystic ovary syndrome, and cancer. We present a novel approach to diagnosing aberrations in glucose utilization including metabolic pathway switching in a disease state.

METHODS—We used a well-defined prenatally exposed glucocorticoid mouse model that results in adult females with metabolic dysfunction. We applied the complementary technologies of nuclear magnetic resonance spectroscopy, and cavity ringdown spectroscopy to analyze serial plasma samples and real-time breath measurements following selective ¹³C-isotope assisted labeling (SIAL). These platforms allowed us to trace metabolic markers in whole animals and identify key metabolic pathway switching in prenatally glucocorticoid-treated animals.

RESULTS—Total glucose flux is significantly proportionally increased through the major oxidative pathways of glycolysis and the pentose phosphate pathway in the prenatally glucocorticoid-treated animals relative to the control animals.

CONCLUSION—This novel diagnostics approach is fast, non-invasive and sensitive for determining specific pathway utilization, and provides a direct translational application in the healthcare field.

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DISCLOSURE STATEMENT Authors F.M.A.P., M.T., and W.P.P. are cofounders of Isomark LLC.

AUTHOR CONTRIBUTIONS Project was initiated by F.M.A.P., who designed the experiments. F.M.A.P., J.A.H and W.P.P. wrote the paper. J.A.H conducted the animal experiments. M.T. wrote the NMR pulse program and F.M.A.P., D.T.H. and M.T. participated in NMR data collection and analysis.

Keywords

glucose; metabolomics; pentose phosphate pathway; glycolysis

INTRODUCTION

Organismal biochemical processes are tightly regulated by their respective enzymatic activities and influenced by hormonal fluctuations and feedback mechanisms. Small molecule metabolites resulting from metabolic processes are sensitive biochemical phenotypes that could be early reporters of a disease condition. Defects in the control of metabolic processes lead, in part, to cancer, aging, diabetes and obesity. A fast, non-invasive method to measure activities of biochemical and metabolic regulatory processes provides a strong foundation for new early diagnostics and therapies for metabolic diseases and cancer.

In normal mammalian cells, a critical metabolic regulatory process concerns glucose metabolism. Glucose oxidation via glycolysis accounts for the majority of carbon flux because it is energetically favorable. The pentose phosphate pathway (PC) includes another oxidative pathway available under aerobic conditions, which is normally reserved for producing NADPH for reductive biosynthetic reactions such as fatty acid and steroid hormone synthesis.

We used a well-defined prenatally glucocorticoid-treated mouse (referenced as cortisol) model to induce metabolic dysfunction. Previous studies on prenatal glucocorticoid exposure indicated a strong correlation between maternal stress and glucocorticoid levels to their adult offspring's metabolic dysfunction including increased visceral adiposity [1-12]. This metabolic dysfunction is associated with an attenuated negative feedback response of the hypothalamic-pituitary-adrenal axis [13-15]. Previously, Chapman *et al.* tested a variety of prenatally administered sex-steroid hormones [16]. They showed that prenatal cortisol treatment in mice, while having the least inhibitory effect on the reproductive system, still presented milder hallmarks of metabolic dysfunction in the female offspring similar to those observed in women with polycystic ovary syndrome (PCOS). We hypothesized that preprogramming of the metabolic dysfunction in the female offspring results from an increased usage of the PC for oxidative catabolism of glucose due to the role that this pathway plays in fatty acid and steroid hormone synthesis.

Katz and Wood originally used differentially labeled glucose with radioactive carbon to study retrospective snapshots of biochemical pathways in normal glucose metabolism in cell-culture, isolated tissues, and perfused organs [17-19]. Katz and Wood described equations to model PC and Embden Meyerhof (EM) usage in cells based on the labeled products from the radioactively labeled glucose substrates [17]. However, they largely neglected recycling of glucose, which makes their equations underestimate the PC usage for *in vivo* scenarios. Christensen et al 2001 developed a new algorithm to account for the effect of recycling of selectively labeled glucose to better estimate the PC contribution in microorganisms [20]. More recently, with the shift away from radioactive isotopes, Eisenreich *et al.* established a method for quantitative assessment of recycling using multiple stable isotopologs to trace carbohydrate metabolism in *Drosophila melanogaster* detected by NMR [21]. Amaro also used NMR to elucidate metabolic phenotypes associated with multidrug resistance in tumor cells through specific stable isotope labeling of glucose [22]. Here, we used glucose labeled with the non-radioactive carbon isotope (^{13}C) in the 1 or 6 positions, in carbon oxidation of glucose during catabolism, to exploit the relative changes in the EM and PC pathway in healthy prenatally vehicle-treated (herein referenced as control) and cortisol mice. We measured metabolic fluxes of ^{13}C and ^{12}C isotopes present in

exhaled breath CO₂ in cortisol and control mice. The changes in breath isotope ratios were confirmed by measuring small molecules plasma (metabolites) by NMR. We present a novel approach to diagnose metabolic pathway switching related to aberrant glucose utilization using selective isotope assisted labeling (SIAL) of breath and plasma biomarkers to trace whole animal metabolism. Our method offers, real-time, non-invasive measurements to determine specific pathway utilization and provides a direct translational application in the healthcare field.

METHODS

Experimental procedures

All animal procedures were approved by University of Wisconsin, College of Letters and Science, Animal Care and Use Committee (Madison, WI, USA).

Reagents

6 β -hydroxycortisol, [1-¹³C]-glucose, [6-¹³C]-glucose, and [U-¹³C]-glucose were from Sigma (Sigma-Aldrich Corp., St Louis, MO, USA). Certified organic sesame oil was from SAFC Supply Solutions (St. Louis, MO, USA). 0.9% saline was from Hospira Corp. (Lake Forest, IL, USA). 6-Aminonicotinamide (6-AN) was from Cayman Chemical Company, Ann Arbor, MI, USA.

Mouse metabolic perturbation

Eleven CD-1 breeding pairs of mice were obtained from Harlan (Indianapolis, IN, USA). Mice were fed AIN-93G diet (Harlan, Indianapolis, IN, USA) and water *ad libitum* during normal housing conditions. All mice were allowed to acclimate for one week prior to mating. A vaginal plug confirmed mating (gestational day, GD, 1). Dams were randomly assigned subcutaneous injections on GD 16 through GD 19 of either 6 β -hydroxycortisol dissolved in ethanol (1.9 mg/kg/day) (1:9 v/v; n=6) and certified organic sesame oil or “vehicle” (n=5). Sesame oil was used as a fat-soluble liquid to minimize the amount of ethanol being injected into a mouse. F1 generation females were weaned and tagged on postnatal day 21 when males were euthanized, because previous studies using this mouse model focused on females [19]. Females used in assays described below were randomly chosen from litters (cortisol, n=3; vehicle, n=3).

Use of selectively labeled glucose to trace metabolites in plasma and in breath

[1-¹³C]-glucose ([1-¹³C]-glc), and [6-¹³C]-glucose ([6-¹³C]-glc) were used as selectively labeled glucose molecules to trace ¹³C-lactate labeling in plasma and ¹³CO₂ in breath to estimate relative use of EM and PC in cortisol and control mice. For injection, each labeled glucose solution was prepared by dissolving 2g (NMR) or 0.2g (CRDS) of glucose in 6 mL medical grade 0.9% saline (1:3 ratio for NMR and 1:30 ratio for CRDS). All solutions were stored at -80°C until use.

Breath sample collection

The CRDS calculates a $\delta^{13}\text{C}$ value which is directly proportional to ¹³CO₂ / ¹²CO₂ and has been previously described [23]. All animals were injected intraperitoneally with one kind of labeled glucose injections: either [1-¹³C]-glucose (cortisol (n=4), vehicle (n=3)); or [6-¹³C]-glucose (cortisol (n=3), vehicle (n=3)). To record a baseline fasting $\delta^{13}\text{C}$ value for individual animals, each animal was first placed in a sealed custom made 1230 cm³ air-tight chamber with aspen bedding with free access to water, and no food for two hours prior to glucose injection. All breath data was collected at the same time of day. Total injection volume in mL was 0.06 times the animal's weight in grams. All animals were used as their

own control and were injected with both species of glucose with a minimum of two weeks between injections. All experiments were run on the CSN-015 CRDS (Picarro, Sunnyvale, CA, USA) using the setup described by Butz et al. [24]. To obtain higher temporal resolution of peak isotopic changes, manual samples of 120 cm³ were taken every 3 min for one hour from some mice following the glucose injection. These samples were placed in Tedlar® gas sampling bags (Sigma-Aldrich Corp., St. Louis, MO, USA) and were run randomly on CRDS. All animals were sampled for two hours pre-injection and four hours post glucose injection. Breath data reported in Table 1 is normalized to the maximum $\delta^{13}\text{C}$ value to align and to compare the maximum values for all animals irrespective of time after glucose administration. Alternatively, the breath data is reported in Fig. 2a,b and Supplementary Fig. 3 as normalized to injection time rather than maxima.

Plasma sample collection

Preparation of plasma samples for NMR—Ice-cold methanol (2:1, v/v) was added to samples before placement at -20°C for 20-30 minutes followed by 5 min centrifugation at 15,000 rpm. The supernatant was then removed from the protein pellet and dried in a speed vacuum overnight. The dried supernatant was then reconstituted by adding a 20 mM phosphate buffer containing 1 mM DSS and 0.1 mM NaF then adjusted to pH 7.4 $\pm$ 0.05.

Collection of NMR spectra

All NMR spectra were collected using a Varian 600 NMR spectrometer equipped with cryogenic probe with the temperature adjusted to 25°C . To obtain one-dimensional (1D) spectra that contained only signals from ^{13}C -bound protons, two different ^1H spectra were collected: one specific to ^{12}C and the other isotopically non-specific ($^{12}\text{C}+^{13}\text{C}$). Each 1D spectrum, ^{12}C -bound ^1H spectrum and $^{12}\text{C}+^{13}\text{C}$ -bound ^1H spectrum, was accumulated for 1028 scans, with an acquisition time of $\sim 400\text{ms}$ (4,096 complex points) and a 3 second repetition delay for a total collection time of 2 hours. In the ^{12}C -bound ^1H spectrum, signals from ^{13}C -bound protons were suppressed by using consecutive low-pass J filters that convert antiphase ^{13}C -bound ^1H magnetization to undetectable double- and zero-quantum coherence [25]. To achieve the best suppression possible for all aliphatic ^{13}C -bound protons (no aromatic ^{13}C -bound protons were expected in our experiments), three consecutive filtering elements with $1/4^1J_{\text{HC}}$ delays tuned to 128 Hz, 134 Hz, and 140 Hz coupling constants were used. Isotopically non-specific spectra ($^{12}\text{C}+^{13}\text{C}$ -bound protons) were collected using the same pulse sequence, but with the carbon transmitter tuned 300 ppm off-resonance to prevent the filtering elements from functioning. This approach ensures that the complementary spectra are collected under identical conditions. $^{12}\text{C}+^{13}\text{C}$ and ^{12}C data was collected in interleaved scans, and both utilize carbon decoupling to minimize spectral complexity. After data collection, ^{13}C -edited data was obtained by taking the difference between the ^{12}C -bound ^1H spectra and the isotopically non-specific ($^{12}\text{C}+^{13}\text{C}$ -bound ^1H) spectra.

For all 1D ^1H spectra, water suppression was achieved by applying a weak saturation pulse (0.1 kHz field strength) of two seconds during the repetition delay and an 80 ms spinlock pulse of 3.5 kHz was used prior to acquisition to suppress fast relaxing signals from large molecules, such as proteins, leaving only peaks from slow relaxing small metabolites.

1D data was then analyzed using Chenomx NMR suite (Chenomx Inc., Edmonton, Alberta, Canada) software to identify relative concentrations of metabolites of interest.

Inhibition of PC with 6-aminonicotinamide

Individual animals (cortisol, $n=3$; vehicle, $n=3$) that were used for breath sample collection and NMR analysis were injected with the specifically labeled glucose species. In addition to

[1-¹³C]-glucose, 6-AN was added to the injection. Animals received 2mg 6-AN/kg body weight with either 3.33% or 33% [1-¹³C]-glc labeling for CRDS or NMR analysis, respectively. The amount of inhibition by 6-AN was assumed constant.

Statistical analysis

Despite the small sample size normality was confirmed by fitting 95% of the data within two standard deviations. Additionally the variance was low enough to produce statistically significant results. The standard error of the mean is reported as error bars on the relevant figures. Two tailed Student's t-Tests assuming type 1 errors were performed using Microsoft Excel and p-values are reported.

RESULTS

Switching to non-lipids substrates using fasting breath biomarkers

The carbon isotope ratio from ¹³CO₂ and ¹²CO₂ reported as the δ¹³C value was determined by CRDS. The δ¹³C value is a quantitative indicator of the endogenous and exogenous source of the carbon substrate being used for metabolism in the body [23].

Fasting δ¹³C values were measured to determine endogenous (basal) substrate consumption in the absence of selective labeling. During normal fasting, after carbohydrate resources are depleted, the substrate source for metabolism switches to lipids causing a significant drop in δ¹³C to a more negative value. The fasting state baseline δ¹³C values prior to glucose isotope labeling showed 2-3 units difference in δ¹³C (p<0.05) between cortisol vs. control animals (Supplementary Fig. 1). Interestingly, the cortisol animals showed baseline δ¹³C values approximately two δ¹³C values higher (−16.44) than the control group (−18.43), indicating utilization of a substrate other than lipid in the cortisol animals.

SIAL application and real-time measurements tracing glucose flux in breath

The selectively labeled glucose, [1-¹³C]-glc or [6-¹³C]-glc, was used to measure the amount of release of labeled and unlabeled CO₂ in breath and to calculate glucose flux through both PC and EM pathways. Each group of fasted animals was injected with each of [1-¹³C]-glc or [6-¹³C]-glc molecules. When the control group was injected with the [6-¹³C]-glc, the baseline δ¹³C value increased to a maximum δ¹³C value of 327.5 (± 5.4) within 40 min, which was significantly higher than the [1-¹³C]-glc, with a maximum δ¹³C value of 235.9 (± 27.4) (Fig. 2a and Table 1).

Conversely, the injection of the cortisol mice with each of [1-¹³C]-glc or [6-¹³C]-glc showed a significant increase in their maximum δ¹³C values from either of the labeled glucose molecules (Fig. 2b and Table 1). Following labeled [6-¹³C]-glc injection, the baseline δ¹³C value for the cortisol mice increased to a maximum δ¹³C value of 394.2 (± 67.5) as compared with [1-¹³C]-glc injection that increased δ¹³C value to 435.1 (± 46.8) (Fig. 2b and Table 1). Furthermore, in the cortisol mice, the maximal δ¹³C values from the labeled glucose molecules were significantly higher than the control group.

The measured δ¹³C values over 240 min (Fig. 2a,b) were integrated to compare the total flux of glucose through EM and PC pathways in the cortisol and control mice (Table 1, and see also Supplementary Material). The integration of δ¹³C values over 4 hours post [1-¹³C]-glc injection for the cortisol mice showed a 29% increase in the integrated δ¹³C values indicating a significant increase in utilization of the PC relative to the control mice (Fig. 2b). Furthermore, [6-¹³C]-glc injection for the cortisol mice showed a 40% increase in the integrated δ¹³C values relative to the control mice indicating a significant increase in total glucose flux through both PC and EM pathways leading to an 11% increase in EM.

However, the ratio of PC to EM within each group remained similar (PC ~ 20-22% and EM ~78-80%).

^{13}C -labeled lactate as a selective marker for metabolic fluxes

Changes in labeled products from each of the selectively labeled glucose precursors, ($[1-^{13}\text{C}]\text{-glc}$, $[6-^{13}\text{C}]\text{-glc}$, or $\text{U-}^{13}\text{C}\text{-glc}$), were compared by NMR to evaluate the relative changes in flux through PC between control and cortisol animals. Figure 3, a-c traces show three different regions of a ^{13}C -edited 1D ^1H spectrum containing only peaks for those protons attached to ^{13}C as selectively labeled reporters. Trace **a** shows lactate and alanine (Ala) regions, whereas traces **b** and **c** refer to glucose spectral regions in the control and cortisol animals two hours post glucose injection. The labeled products were identified to be specific to lactate and alanine (Fig. 3a-c), and acetate (not shown). The level of the labeled ^{13}C -lactate derived from $[1-^{13}\text{C}]\text{-glc}$ was significantly higher than the labeled ^{13}C -lactate derived from $[6-^{13}\text{C}]\text{-glc}$ in the controls (Fig. 3a, and Supplementary Fig.2B-E). The less targeted $\text{U-}^{13}\text{C}\text{-glc}$ labeling approach generated significantly higher amounts of ^{13}C -lactate in the control than either $[1-^{13}\text{C}]\text{-glc}$ or $[6-^{13}\text{C}]\text{-glc}$ (Fig. 3a).

The ratio of ^{13}C -lactate concentration derived from $[1-^{13}\text{C}]\text{-glc}$ to the total $^{13}\text{C}+^{12}\text{C}$ -lactate concentration was 0.06 (± 0.007) in the cortisol animals compared to 0.09 (± 0.006); $p=0.03$ in the control mice. The decreased $[1-^{13}\text{C}]\text{-glc}$ derived lactate level after two hours indicated a ~30% increase in the flux of labeled glucose through the PC in the cortisol mice (Fig. 3a). This is consistent with total flux calculated from breath $\delta^{13}\text{C}$ values suggesting a 29% increase in PC (Fig. 2b).

The decreased lactate level was also confirmed with $\text{U-}^{13}\text{C}\text{-glucose}$ tracing, which indicates that $\text{U-}^{13}\text{C}\text{-glc}$ derived lactate was also reduced by ~30% in the cortisol animals compared to the controls. However, the $[1-^{13}\text{C}]\text{-glc}/^{13}\text{C}\text{-lactate}$ ratio for all animals was calculated to be ~ 5:1 whereas the $\text{U-}^{13}\text{C}\text{-glc}/^{13}\text{C}\text{-lactate}$ ratio was ~1:1 suggesting a higher flux from $\text{U-}^{13}\text{C}\text{-glc}$ to generate ^{13}C -lactate derived molecules compared to $[1-^{13}\text{C}]\text{-glc}$.

SIAL glucose to trace ^{13}C -recycling in plasma by NMR

The glucose region of the NMR spectrum between 3.2-3.9 ppm (Fig. 3c, Supplementary 2AB) showed significant scrambling of ^{13}C from $[1-^{13}\text{C}]\text{-glc}$ to positions 2, 3, 4, 5 and 6 with the less recycling going to position C4 (Supplementary Fig. 2B-E). Conversely, recycling from $[6-^{13}\text{C}]\text{-glc}$, generated less scrambling (Supplementary Fig. 2A-E) compared to $[1-^{13}\text{C}]\text{-glc}$ to positions 1, 4 and perhaps 3. Uniform labeling with $\text{U-}^{13}\text{C}\text{-glucose}$ ($\text{U-}^{13}\text{C}\text{-glc}$) resulted in larger quantities of similarly labeled metabolites (pyruvate, acetate, lactate, and alanine), while recycling of ^{13}C to other positions could not be clearly distinguished because of the uniform labeling (Fig. 3b-c and Supplementary Fig. 2C). However, a smaller amount of ^{13}C -label at position 1 was noticeable relative to other positions (~30% less). The total distribution in α : β ratio of ^{13}C -labeling remained constant (~ 40:60) among all protons attached to ^{13}C -carbons from each type of labeled glucose. Additionally, the level of ^{13}C scrambling did not change in both control and cortisol animals from $[1-^{13}\text{C}]\text{-glc}$ or $[6-^{13}\text{C}]\text{-glc}$ (Fig. 3c).

6-AN, an inhibitor of PC

The increased glucose flux especially through the PC in the cortisol mice was further confirmed by co-administration of $[1-^{13}\text{C}]\text{-glc}$ and a known PC inhibitor, 6-AN (Supplementary Fig. 3). Due to the acute toxicity of 6-AN (LD_{50} 35mg/kg body weight for mice) [26], it was only possible to partially knockdown the PC pathway in live animals with a very small dose of 6-AN (2mg/kg body weight) without sacrificing any animals. Although using a small dose of 6-AN allows for only partial inhibition of PC in the cortisol mice, it is

still significant for validation of our proposed model of metabolic pathway disruption (Supplementary Fig. 3). Control animals experienced a significant increase in maximum breath $\delta^{13}\text{C}$ value (Table 1) from 235.9 to 379.6 ($p=0.03$) following $[1-^{13}\text{C}]\text{-glc}$ administration without and with 6-AN, respectively, indicating increased glucose flux through the EM. However, cortisol animals showed no significant difference in the maximum $\delta^{13}\text{C}$ value following administration of 6-AN ($p=0.65$) (Table 1) supporting an upper limit for glucose flux through both EM and PC in these animals. The traces depicted in Supplementary Fig. 3 are shown as real-time $\delta^{13}\text{C}$ values averaged at a given time point and are relative to the injection time.

However, since co-administration of $[1-^{13}\text{C}]\text{-glc}$ and 6-AN caused individuals to have delayed response times (12-26 minutes) in the maximum $\delta^{13}\text{C}$ value relative to injection time (Supplementary Fig. 3) the values reported in Table 1 were normalized to the maximum $\delta^{13}\text{C}$ values irrespective of injection time.

Co-administration of 6-AN and $[1-^{13}\text{C}]\text{-glc}$ in both control and cortisol animals showed a significant change in labeled lactate in plasma (data not shown). Lactate from $[1-^{13}\text{C}]\text{-glc}$ relative to the total amount of metabolism was significantly decreased from 18% to 13% after 6-AN inhibition in control mice suggesting a 5% reduction in labeled lactate formation. Similarly, cortisol mice showed a 5% reduction in labeled lactate formation with 13% before and 8% after inhibition, respectively. However, the initial ^{13}C -lactate level in the cortisol mice was significantly less compared to the control, indicating that the glucose flux through EM is already maximal in these animals and additional glucose could not be diverted to the EM pathway.

DISCUSSION

Our results provide a proof of principle for detecting biomarkers of metabolic dysfunction through tracing specifically labeled pathways in serum and breath of animals using SIAL technology. The use of a well-defined prenatally glucocorticoid-treated mouse model was essential to diagnose metabolic perturbations in a PCOS-like metabolic dysfunction.

The novel combination of SIAL technology in conjunction with NMR and CRDS identifies key contributing metabolites that could be otherwise concealed using standard non-targeted metabolomics approach. Through the bridging of these important technologies, we were able to trace preferential use of the key metabolic pathways and their relative fluxes. Additionally, this study confirms that both breath and plasma are sensitive reporters of metabolic changes that underlie the foundation of prenatally induced metabolic disease. This technology can be directly applied to other pathologies involving aberrant glucose metabolism such as infection [24], cancer [27], diabetes mellitus type II, polycystic ovary syndrome [28], and obesity [29], providing clinical medicine with a reliable, easily applied methodology to diagnose disease and/or monitor recovery.

In this study, the use of ^{13}C edited ^1H spectra method is advantageous compared to direct detection of ^{13}C resonances because it produces a reliable estimate of the relative amount of ^{13}C species versus endogenous ^{12}C species in the samples from spectra for ^{13}C species, ^{12}C species or both combined. The ^{13}C direct detection method, conversely, offers only the ^{13}C species present, without information regarding the ^{12}C species.

Application of NMR and SIAL to whole animal systems allows measuring fluxes of ^{13}C -labels to selected pathways such as EM or PC. However, through shuffling and recycling of label via gluconeogenesis and PC pathways, the ^{13}C -labeling distribution could not be accurately measured. Recycling of ^{13}C -label caused by increased reshuffling of ^{13}C -from $[1-^{13}\text{C}]\text{-glc}$ to other positions results in decreased plasma ^{13}C -lactate level derived from

position 1, thus underestimating the flux through PC. The NMR data shows reduced recycling when [6-¹³C]-glc (10-30%) is used compared to the [1-¹³C]-glc position (26-40%), thus diminishing the sensitivity towards precise measurements of splitting between PC and EM. While the role of recycling had been previously examined in microorganisms and their contributions had been used in modeling fluxes through PC and EM [20,30], due to its complexity in whole animals the relative changes in PC are most relevant to measured values by NMR. Furthermore, the recycling flux for each selectively labeled substrate is similar between the control and the cortisol animals as indicated by NMR. Thus, to exclude the recycling contribution to EM and PC pathways, we report relative changes in total flux between the control and the cortisol animals.

Decreased levels of both labeled lactate and labeled acetate in plasma from cortisol mouse vs control following [1-¹³C]-glc treatment can be attributed to the enhanced flux through the PC (30%) and likely contributing to the downstream lipid biosynthesis in cortisol animals. Consequently, the higher PC glucose flux results in production of more unlabeled acetate downstream as a result of the loss of ¹³CO₂ from the position 1 of [1-¹³C]-glc in cortisol animals (Fig. 1). On the other hand, control animals preferentially use the EM pathway, resulting in higher levels of labeled lactate following [1-¹³C]-glc treatment. As expected, the [6-¹³C]-glc derived lactate shows no difference in either group because the labeled carbon is not lost in the PC as carbon dioxide.

The use of breath measurements is essential in real-time flux measurements because they are fast, non-invasive, cumulative from all molecular sources and can be measured continuously. Breath stable isotopes have been previously applied to metabolic [31-32] and nutritional statuses [33]. Breath measurements by CRDS are particularly useful to determine the total amount of glucose flux through both PC and EM pathways because of its high sensitivity measuring ¹³CO₂/¹²CO₂ ratios [24]. The endogenous ¹²CO₂ concentration in breath is typically 4% and is diluted with room air (0.039% CO₂ of which 1.1% is ¹³CO₂) to a final concentration of 1% before entering the CRDS chamber. The change in observed δ¹³C value is exclusively derived from the ¹³C-labeled injection and is relative to a constant amount of ¹²C thus could be a sensitive indicator of glucose flux through oxidative pathways. Breath measurements suggest an overall significantly increased glucose flux through the EM (11%) and the PC (29%) pathways in cortisol animals. The higher glucose flux through PC than EM observed in cortisol animals suggests a major metabolic switching from EM to PC in a PCOS metabolic-like syndrome due to excess NADPH resulting from the increased PC usage. This reducing power is essential for biosynthetic reactions that lead to increased levels of cholesterol, steroid hormones, and lipids, and contributes to the metabolic phenotypes seen in PCOS and obesity.

In summary, the combined NMR data from biofluids, SIAL and CRDS measurements from breath are tools that can be applied for the first time in humans to 1) identify metabolites that can serve as rapid diagnostic indicators of metabolic dysfunction, 2) provide a better estimation of fluxes through the metabolic pathways involved in the dysfunction and 3) suggest more targeted therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

6-AN	6-Aminonicotinamide
CRDS	cavity ringdown spectroscopy
CO₂	carbon dioxide
δ¹³C	delta value
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
EM	Embsden Meyerhof
GD	gestational day
glc	glucose
G6PD	glucose-6-phosphate dehydrogenase
NADPH	nicotinamide adenine dinucleotide phosphate
NTP	non-triose pathways
NMR	nuclear magnetic resonance
PC	pentose phosphate pathway
SIAL	selective isotope assisted labeling
FA	fatty acid
NaF	sodium fluoride

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Figure 1. Proposed carbon fate following injection with [1-¹³C]-glucose

Schematics of different fates of labeled carbon in glucose metabolism following injection with [1-¹³C]-glucose in: **(a)** control mice **(b)** cortisol mice. Arrow width indicates the relative amount of pathway usage. Cortisol mice show significant increase in usage of the pentose phosphate cycle (PC) relative to Embden Meyerhof (EM) indicating an overall increase in glucose flux relative to control mice. Light gray boxes indicate CO₂ detection in breath by CRDS. Dark gray boxes indicate small metabolites detection in plasma via NMR.

Figure 2. SIAL labeling and tracing of ^{13}C -stable isotope biomarkers in breath by CRDS
 Traces are $\delta^{13}\text{C}$ values from exhaled carbon dioxide shown for: (a) control mice, and (b) cortisol animals. The blue traces in both panels are the $\delta^{13}\text{C}$ values following injection with $[1-^{13}\text{C}]$ -glucose (vehicle (n=3), cortisol (n=4)). The red traces in both panels are the $\delta^{13}\text{C}$ values following injection with $[6-^{13}\text{C}]$ -glucose (vehicle (n=3), cortisol (n=3)). Animals prenatally treated with cortisol show elevated glucose flux. All traces are with respect to the injection time. The traces reflect the average $\delta^{13}\text{C}$ values every fifteen minutes over a period of six hours for all animals relative to the injection time and are not corrected for matching maximum $\delta^{13}\text{C}$ values. A time delay for peak $\delta^{13}\text{C}$ value exists following treatment with $[6-^{13}\text{C}]$ -glucose in the cortisol mice. The control mice reach a peak $\delta^{13}\text{C}$ value at 0.57 hours post injection whereas the prenatally cortisol-injected mice reach a peak $\delta^{13}\text{C}$ value at 0.75 hours post injection. The unit of measurement for the $\delta^{13}\text{C}$ values reported is ‰ (parts per mil). Error bars represent the SEM. $\delta^{13}\text{C}$ values normalized to the maximum $\delta^{13}\text{C}$ value are reported in Table 1.

Figure 3. SIAL tracing of ^1H attached to ^{13}C -lactate by NMR

Traces **a-c** of selected NMR regions after ^{13}C -glucose enrichment in control and cortisol female mice. The plasma was obtained 2 h after injection with one of three different labeled glucose precursors: $[1-^{13}\text{C}]$ -glucose, $[6-^{13}\text{C}]$ -glucose, or ^{13}C -U-glucose as indicated in the figure. The spectra shown contain only peaks from ^1H that are attached to ^{13}C . These spectra were obtained by subtracting a ^{13}C -filtered spectrum (containing only protons attached to ^{12}C) from the corresponding unfiltered experiment (containing both ^{13}C and ^{12}C species). Panel (**a**) shows lactate and alanine peaks. Cortisol animals show a decrease in the amount of ^{13}C -lactate derived from both $[1-^{13}\text{C}]$ -glucose and ^{13}C -U-glucose. Panel (**b**) shows ^1H - $[1-^{13}\text{C}]$ -glucose assigned peaks indicating a decrease in plasma ^{13}C -glucose in cortisol mice. Panel (**c**) depicts recycling of ^{13}C -label to the rest of ribose protons (^1H - ^{13}C) in designated spectral region, * indicates peak arising from methanol. Assignments for ribose protons are shown in Supplementary Fig. 2A,B. Similar recycling is observed between the control and cortisol animals in each of labeled glucose.

Table 1 $\delta^{13}\text{C}$ Values following labeled glucose injection

Treatment	Glucose Label	Max $\delta^{13}\text{C}$ Value*	p-value	$\delta^{13}\text{C}$ Value Integration
Control	C-1 (n=3)	235.9 (27.4)	*0.03	516.9
	C-6 (n=3)	327.5 (5.4)		645.7
	C-1+6-AN (n=3)	379.6(32.4)	#0.028	722.5
Cortisol	C-1 (n=4)	435.1 (46.8)	*0.63	703.5
	C-6 (n=3)	394.2 (67.5)		903.0
	C-1+6-AN (n=3)	402.2 (45.8)	#0.65	743.7

* $\delta^{13}\text{C}$ values are normalized to the maximum value of each individual irrespective of injection time. SEM is noted in parentheses.

* Comparison of C-1 to C-6 within treatment group.

Comparison of C-1 to C-1 +6-AN within treatment group. All units are %. Integration values correspond to curves reported in Figs 2 and Fig 3S.