Plasma Metabolomic Profiles Reflective of Glucose Homeostasis in Non-Diabetic and Type 2 Diabetic Obese African-American Women

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Abstract

Insulin resistance progressing to type 2 diabetes mellitus (T2DM) is marked by a broad perturbation of macronutrient intermediary metabolism. Understanding the biochemical networks that underlie metabolic homeostasis and how they associate with insulin action will help unravel diabetes etiology and should foster discovery of new biomarkers of disease risk and severity. We examined differences in plasma concentrations of >350 metabolites in fasted obese T2DM vs. obese non-diabetic African-American women, and utilized principal components analysis to identify 158 metabolite components that strongly correlated with fasting HbA1c over a broad range of the latter (r = −0.631; p < 0.0001). In addition to many unidentified small molecules, specific metabolites that were increased significantly in T2DM subjects included certain amino acids and their derivatives (i.e., leucine, 2-ketoisocaproate, valine, cystine, histidine), 2-hydroxybutanoate, long-chain fatty acids, and carbohydrate derivatives. Leucine and valine concentrations rose with increasing HbA1c, and significantly correlated with plasma acetylcarnitine concentrations. It is hypothesized that this reflects a close link between abnormalities in glucose homeostasis, amino acid catabolism, and efficiency of fuel combustion in the tricarboxylic acid (TCA) cycle. It is speculated that a mechanism for potential TCA cycle inefficiency concurrent with insulin resistance is “anaplerotic stress” emanating from reduced amino acid-derived carbon flux to TCA cycle intermediates, which if coupled to perturbation in cataplerosis would lead to net reduction in TCA cycle capacity relative to fuel delivery.

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Introduction

Type 2 diabetes mellitus (T2DM) is a progressive disease in which increasingly poor insulin sensitivity and hyperinsulinemia precede, typically for several years, the onset of frank diabetes [1]. The central feature of the insulin resistance involves reduced insulin-mediated glucose uptake and metabolism, particularly in skeletal muscle. However, evidence also suggests dysregulated fatty acid metabolism and tissue lipid accumulation as being associated with the development of insulin resistance and T2DM [2;3]. Furthermore, in human obesity and in some obese, diabetic animal models an elevation of circulating branched chain amino acid (BCAA) concentrations has been reported [4–9]. Thus, insulin resistance progressing to T2DM is marked by a broad perturbation of macronutrient intermediary metabolism.

Excessive cellular accumulation of lipids in muscle [10–17], liver [18], and adipocytes [19] is associated with insulin resistance in those tissues. Lipotoxicity in pancreatic β-cells also is proposed as a factor leading to loss of β-cell function in T2DM [20]. In skeletal muscle specifically, lower long-chain fatty acid (LCFA) oxidation in the fasted state and a blunted increase of carbohydrate oxidation in response to increased insulinemia (“metabolic inflexibility”) is a common feature of pre-diabetes and T2DM [21]. Insulin resistance may be accompanied by a greater mismatch between muscle LCFA delivery and tissue oxidative capacity, leading to accumulation of by-products of incomplete LCFA oxidative catabolism [22]. Consistent with this model, acylcarnitines resulting from incomplete LCFA oxidation were more prevalent and elevated in the plasma of type 2 diabetic women compared to non-diabetics [23]. However, the tissue source of these metabolites cannot be pinpointed from plasma patterns alone. The molecular factors linking inefficient LCFA catabolism to impaired insulin action remain controversial, but may include build-up of cytosolic ceramide and diacylglycerol (DAG) that inhibit Akt/PKB and activate PKC enzymes, respectively [see reviews by [24–26]]. Increased tissue exposure...
to saturated fatty acids can activate pro-inflammatory cascades associated with insulin resistance via toll-like receptors 2 and 4, and we reported that at least some acylcarnitines elevated in T2DM plasma can trigger NFkB-driven gene expression, suggesting that these metabolites have pro-inflammatory properties as well [23].

Understanding the biochemical networks underlying metabolic homeostasis and their association with insulin sensitivity will help to clarify diabetes etiology, and should foster the discovery of new biomarkers of disease risk and severity. It would be particularly useful to identify metabolite signatures specific to muscle LOFA oxidation considering the importance of lipid metabolism in this tissue to whole-body insulin sensitivity. However, there is a paucity of comparative experimental models in which β-oxidation is altered exclusively or predominantly in muscle cells. To address these problems, we have applied metabolomics platforms to compare plasma metabolite patterns in weight-matched obese non-diabetic and T2DM African-American women, with or without an uncoupling protein 3 (UCP3) g/a missense polymorphism that results in substantial reductions in whole-body LOFA oxidation [27]. Considering that UCP3 is essentially muscle-specific in humans, examination of metabolic patterns in persons harboring the UCP3 g/a polymorphism holds promise to uncover muscle-specific moieties reflective of altered muscle β-oxidation. The archived plasma samples examined herein are identical to those used in our recent report of acylcarnitine profiles in this cohort [23], and this complementary work extends the metabolite coverage to >400. The results indicate that variances in a unique subset of metabolites successfully discriminate non-diabetics from T2DM subjects. The metabolite patterns highlight that indices of poor blood sugar control, and markers of inefficient TCA cycle function, strongly correlate with increased plasma BCAA concentrations, which we propose reflects disruption of normal amino acid catabolism and hence imbalanced anaplerosis. The data also point to a potential role of mitochondrial UCP3 in regulation of glutamate/α-ketoglutarate/butanoyl-CoA metabolism.

**Methods**

**Human Volunteers and Blood Plasma Collection**

Comprehensive details regarding the study cohort and sample collection were previously-described [23]. Briefly, archived plasma samples derived from body mass index- (BMI) and age-matched overweight to obese type 2 diabetic (n = 44) and non-diabetic (n = 12) Gullah-speaking African-American women with or without a UCP3 g/a missense polymorphism were evaluated. Volunteers were recruited as part of the Project SuGAR study described in detail elsewhere [28–31]. Considering that this group is of a single sex, displays an extraordinarily low genetic admixture, lives in a relatively small geographical space, and has a common dietary intake pattern, we anticipate that the cohort is well-suited for metabolomics studies since biological metabolite signal-to-noise should be low. Ethics Statement: Studies were approved by the Institutional Review Boards of the Medical University of South Carolina, University of Alabama at Birmingham, and the University of California, Davis, and all participants provided written informed consent. Blood was collected by arm venipuncture between 08:00–09:00 into EDTA-treated collection tubes after an overnight fast (no food or drink since 20:00 the night before). Plasma was frozen at −20°C for 1–7 days before transport to −80°C freezers for longer-term storage. Volunteers were asked to avoid unusual activity and intentional exercise in the 3 days leading up to the study, and were instructed to continue to eat their habitual diet without unusual deviations. Patients with T2DM did not take doses of oral agents on the evening before and on the morning of study. Patients treated with insulin could take regular or rapid acting insulin at dinner the night before the study but were instructed to withhold any intermediate- or long-acting insulin on the evening before, and to avoid insulin injections on the morning of the study.

**Metabolite Analysis**

Plasma samples for metabolomics assays were thawed on ice, aliquoted, re-frozen on dry ice, and stored at −80°C prior to delivery to the Fiehn lab. Plasma aliquots (15 μL) were extracted and derivatized as reported previously [29] using 1 mL of degassed acetonitrile/isopropanol:water (3:3:2; v/v/v) at −20°C, centrifuged and decanted with subsequent evaporation of the solvent to complete dryness. A clean-up step with 500 μL acetonitrile/water (1:1; v/v) removed membrane lipids and triglycerides and the supernatant was dried down again. A set of 13 C8–C30 fatty acid methyl ester internal standards were added and samples were derivatized by 10 μL methoxyamine hydrochloride in pyridine followed by 90 μL MSTFA (1 mL bottles, Sigma-Aldrich) for trimethylsilylation of acidic protons. A Gerstel MPS2 automatic liner exchange system (Mulheim an der Ruhr, Germany) was used to inject 0.5 μL of sample at 50°C (ramped to 250°C in splitless mode with 25 s splitless time). Analytes were separated using an Agilent 6890 gas chromatograph (Santa Clara, CA) equipped with a 30 m long, 0.25 mm i.d. Rtx5Sil-MS column with 0.25 μm 5% diphenyl film and additional 10 m integrated guard column (Restek, Bellefonte PA). Chromatography was performed with constant flow of 1 mL/min while ramping the oven temperature from 50°C for to 330°C with 22 min total run time. Mass spectrometry was done by a Leco Pegasus IV time of flight mass spectrometer (St. Joseph, MI) with 280°C transfer line temperature, electron ionization at −70eV and an ion source temperature of 250°C. Mass spectra were acquired from m/z 85–500 at 17 spectra s−1 and 1850 V detector voltage. Result files were exported to our servers and further processed by our metabolomics BinBase database [32]. All database entries in BinBase were matched against the Fiehn mass spectral library of 1,200 authentic metabolite spectra using retention index and mass spectrum information or the NIST05 commercial library. Identified metabolites were reported if present within at least 50% of the samples per study design group (as defined in the SetupX database) [33]. Peak heights of quantifier ions defined for each metabolite in BinBase were normalized to the sum intensities of all known metabolites and used for statistical investigation. External 5-point calibration curves established with quality control mixtures containing 30 metabolites controlled for instrument sensitivity. Each chromatogram was further controlled with respect to the total number of identified metabolites and total peak intensities to ensure that outliers did not confound the subsequent statistical analysis.

**Statistical Analyses**

A mixture of univariate and multivariate statistics were applied to the investigation of changes in this study. Differences in mean plasma analyte concentrations between subjects with different UCP3 genotypes or diabetic status were initially evaluated using unpaired Student’s t-tests with multiple comparison adjustments made using the method of Benjamini and Hochberg [34]. A false discovery rate (FDR) of 20% (i.e., α = 0.2) was applied. Principal components analyses (PCA) were used to independently assess metabolites that segregated with genotype and diabetic status. Specifically, all detected metabolites were used as input variables, and principal components were ranked on significant differences
between scores of sample classifications. The PCA was performed using the Microsoft Excel add-in developed by the Bristol Centre for Chemometrics, University of Bristol, UK (http://www.chm.bris.ac.uk/org/chemometrics/chemometrics.html). The Pearson’s correlation statistic was used to determine relationships between select metabolites (PrismGraph, GraphPad, San Diego, CA). All results are presented as means ± SEM, and P<0.05 was considered statistically significant.

**Results**

Over 700 discrete signals were detected using GC-TOF mass spectrometry for each plasma sample. After applying the BinBase database filtering, 366 metabolites passed stringent analytical quality control measures, and used for comparisons of diabetic vs. non-diabetic and UCP3 g/g vs. UCP3 g/a plasma metabolomic profiling. The identities of these metabolites are provided in Table S1 (genotype comparisons) and Table S2 (diabetes comparisons). Metabolites lacking full structural identification (“unknowns”) are unambiguously described by BinBase (BB) numbers and full mass spectra, quantifier ions and retention indices. These data are publicly available and queryable against all 24,000 samples in BinBase (http://eros.fiehnlab.ucdavis.edu:8080/binbase-compound/).

**Plasma Metabolite Profiles in Non-Diabetic UCP3 g/g and g/a Polymorphs**

In non-diabetic persons harboring the missense g/a UCP3 allele, although there were 35 metabolites with mean plasma concentrations at least 50% higher vs. g/g subjects (Table S1), the difference in only 2 metabolites achieved statistical significance assessed by unpaired two-tailed t-tests (BB2298860 and BB219174; Table 1). In contrast, 14 of 18 plasma metabolites reduced by at least 50% in g/a polymorphs were significantly different compared to g/g subjects (Table 1). However, changes did not achieve statistical significance after applying a 20% FDR correction (see Methods). Regardless, it is notable that concentrations of 2-oxoglutarate (2-ketoglutarate) and glutamate were both reduced in non-diabetic subjects harboring the g/a allele. In addition, our previous report of a 36% reduction in plasma lactate concentration in non-diabetic g/a subjects measured by standard clinical chemistry analyses [23] was confirmed by mass spectral analysis (Table S1). Most metabolites that were significantly altered in non-diabetic g/a individuals vs. g/g individuals remain unidentified (Table 1 and Table S1).

**Plasma Metabolite Comparisons in Non-Diabetic and Type 2 Diabetic Subjects**

As expected, comprehensive metabolomics analysis of diabetic vs. non-diabetic plasma revealed significantly increased concentrations of glucose, long-chain fatty acids (LCFAs: oleic, palmitoleic, palmiatic), and the ketone body 3-hydroxybutyrate (3-hydroxybutyrate) in T2DM subjects (Table 2). The mean plasma concentrations of 36 metabolites increased ≥50% in T2DM subjects vs. non-diabetics (Table S2). A total of 59 plasma metabolites were decreased in T2DM subjects: 18 metabolites were reduced by at least 25% in diabetics vs. non-diabetics, and 1 of these had a concentration >50% lower in T2DM (BB261134; Table S2). All metabolites that were significantly different when comparing diabetic vs. non-diabetic subjects passed the 20% FDR threshold. Most metabolites altered in diabetics remain unidentified in terms of chemical nomenclature; known metabolites are listed in Table 2.

Of the metabolites that differed in T2DM subjects, it was notable that plasma leucine concentration was significantly increased by ~50% (Table 2), and its initial catabolic metabolite, 2-ketocaproic acid (2-ketocaproate), was significantly increased by ~27% (Table 2). Mean plasma valine concentration was ~20% higher in type 2 diabetic subjects vs. non-diabetics (Table S2), but this difference was not statistically significant. When leucine and valine were each considered in terms of their enrichment in the total plasma amino acid pool (expressed as a % of the total summed quantifier ion peak heights of all detected amino acids), the diabetes-related increases in these BCAAs were even more apparent and statistically significant for both leucine % and valine % (Figure 1). Leucine % enrichment increased concurrent with worsening blood sugar control since degree of enrichment correlated significantly with hemoglobin A1c (HbA1c%; Figure 2A). The relationship between valine % and HbA1c was less robust (Figure 2B).

Using results from our previous study of plasma acylcarnitine patterns in these same samples [23], both leucine and valine % enrichment in the plasma amino acid pool were found to correlate strongly (p<0.001) with acylcarnitine concentration (Figure 2C and 2D). It can also be seen that increasing valine % enrichment is coincident with a reduction in relative concentrations of propionylcarnitine, a marker of the valine catabolic product propionyl-CoA; these patterns are associated with increasing acylcarnitine concentrations (Figure 3).

To identify the specific plasma metabolites with distributions most affected by subjects’ diabetes status, we first employed PCA, an unsupervised multivariate analysis approach, and considered four principal components (PC) dimensions. Metabolite variation in PC dimensions 2 and 3 (PC2, PC3) explained 16.8% of the variance between groups, and the differences between diabetic vs. non-diabetic PC scores were highly significant (P<0.001 for both PC2 and PC3; data not shown). A principal components (metabolites) selection criterion for diabetic and non-diabetic group discrimination was set for components with loadings (i.e., variance contribution) ≥1 standard deviation from the mean loading value in each of the discriminating PC2 and PC3 dimensions. The 158 metabolic components emerging from this approach were used in a secondary PCA to further refine the principal components that most accurately predict the type 2 diabetic phenotype (this analysis did not include glucose since this metabolite defines diabetes status). In this secondary PCA, PC1 explained 40% of the group variance, and the mean PC1 scores in diabetics and non-diabetics were significantly different (p<0.0001), indicating successful phenotype discrimination. An additional 18.3% of the total group variance was accounted for in the PC2 dimension, and the mean PC2 scores in diabetics and non diabetics were significantly different (p<0.0001). The PC1 and PC2 scores were thus plotted for each of the diabetic and non-diabetic subjects to visualize the magnitude of separation of the groups (Figure 4): excellent separation of diabetic and non-diabetic subject cohorts was achieved. Loadings for each of the discriminating factors may be found in Table S3; metabolites with loadings at the highest and lowest extremes in PC1 and PC2 are those with variances most strongly impacting the phenotype separation in the PC1 and PC2 dimensions in Figure 4. The PC1 scores derived from the selected metabolites were highly correlated with the degree of blood sugar control (blood HbA1c%), indicating a significant association between person-to-person differences in these metabolites and the diabetic phenotype (Figure 5).

**Discussion**

Type 2 diabetes mellitus is a disease of abnormal intermediary metabolism of an array of nutrients including glucose. For
instance, the importance of lipid metabolism in diabetes was highlighted in the seminal studies by Randle et al. indicating that muscle glucose utilization can be inhibited by increased provision of long chain fatty acids (LCFAs) [35]. Later perspectives from McGarry, Kelly and others pointed out that abnormal fatty acid oxidation and ectopic tissue lipid accumulation are at the core of insulin resistance and T2DM genesis (see [2;21]), and Unger et al. have implicated lipotoxicity in pancreatic β-cell dysfunction [3;20]. There is now strong evidence that incomplete or inefficient LCFA β-oxidation accompanies insulin resistance and T2DM [22;23]. The underlying mechanisms for this association remain controversial, but it appears that sub-optimal whole-tissue tricarboxylic acid (TCA) cycle function in some tissues contributes to the metabolic phenotype in the insulin-resistant state [36–40]. Thus, to fully understand the metabolic landscape of T2DM, it is necessary to comprehensively determine how multiple pathways change in this condition and to evaluate how disparate pathways interconnect. Efforts to identify specific metabolites associated with T2DM and insulin resistance hold promise in developing clinically facile, predictive diagnostic and prognostic tools to predict diabetes risk, to determine disease stage, elucidate causal mechanisms, and to evaluate the efficacy of modalities aiming to thwart T2DM.

We have taken advantage of advancements in analytical chemistry and informatics tools to determine diabetes-associated patterns of >350 plasma metabolites encompassing carbohydrate, lipid, amino acid, purine, and organic acid classes. These results complement and extend our previous results regarding plasma acylcarnitines in the same subjects [23]. Marked differences in the plasma metabolome of diabetic and non-diabetic obese African-American women were observed, and identified a set of specific metabolites whose variability was strongly correlated with HbA1c, an index of long-term blood sugar control. These metabolites, therefore, appear to be good candidate biomarkers of blood sugar control and may provide insights into metabolic disease etiology. In at least some cases, differentially-abundant plasma metabolites appear to have resulted from hyperglycemia and increased flux of excess glucose toward secondary conversion pathways: i.e., increased fasting plasma fructose concentrations in T2DM subjects appear to have resulted from hyperglycemia and increased flux of excess glucose toward secondary conversion pathways: i.e., increased fasting plasma fructose concentrations in T2DM subjects are indicative of fructose generation (Figure 4), and elevated gluconic acid and xylose (Table 2). The concentrations of less than 10% of the measured metabolites in the current study displayed significant differences of 2-fold or greater when comparing T2DM vs. non-diabetic subjects, and these metabolites might be some of the strongest biomarkers of metabolic health status. Additional potential candidate diabetes biomarkers emerged from PCA analysis, including 93 metabolites for which variance-derived loading values were >1 standard deviation from the mean (Table S3). The PCA metabolite loading value patterns (Table S3) revealed that many metabolites reflecting the diabetic phenotype discovered here await identification since their retention times and m/z ratios did not match standards available to the investigators at the time of analysis. Furthermore, metabolite loading values in both PC1 and PC2 indicated that separation of diabetic and non-diabetic metabolites involved variation in several chemical classes, viz. select carbohydrate derivatives (i.e., fructose, gluco-

### Table 1. Plasma metabolites with significantly-altered concentrations in non-diabetic obese African-American women harboring a UCP3 g/a missense allele.

| Pathway                                           | Relevant Metabolic Pathway | g/a to g/g Ratio | g/g genotype (n = 6) | g/a genotype (n = 6) |
|----------------------------------------------------|---------------------------|-----------------|---------------------|---------------------|
| Increased in non-diabetic g/a:                     |                           |                 |                     |                     |
| BB226860                                           | 2552 ± 669                |                 |                     | 5851 ± 1302         |
| BB219174                                           | 6966 ± 988                |                 |                     | 10253 ± 1074        |
| Decreased in non-diabetic g/a:                      |                           |                 |                     |                     |
| BB223521                                           | 3569 ± 340                |                 |                     | 2516 ± 318          |
| phosphoric acid                                    |                           |                 |                     | 1065028 ± 88171     |
| BB223506                                           | 4884 ± 343                |                 |                     | 3288 ± 483          |
| BB281189                                           | 432579 ± 45901            |                 |                     | 285112 ± 14032      |
| inulobiose                                         | 1223 ± 152                |                 |                     | 795 ± 87            |
| BB228147                                           | 579 ± 67                  |                 |                     | 374 ± 49            |
| BB211382                                           | 36484 ± 3617              |                 |                     | 21651 ± 3493        |
| cysteine                                           | 24519 ± 4380              | 13827 ± 1928    |                     | 1.005               |
| 2-oxoglutarate (α-ketoglutarate)                   |                           |                 |                     | 0.55**              |
| BB281112                                           | 18342 ± 2886              |                 |                     | 9198 ± 2265         |
| BB281444                                           | 3874 ± 659                | 1910 ± 524      |                     | 0.50                |
| BB239966                                           | 2358 ± 418                | 1158 ± 308      |                     | 0.49**              |
| glutamic acid (glutamate)                          | 48146 ± 7473              | 21048 ± 1427    |                     | 0.44**              |
| BB22169                                            | 25526 ± 4397              | 10743 ± 4133    |                     | 0.42               |

Values are quantifier peak height means ± SEM; see Supplemental Table S1 for full list of metabolites including those whose concentration differences were not statistically significant:

* p < 0.05;

** p < 0.01 (unpaired t-test).

Note that after application of a 20% false discovery rate (see Methods) these differences did not achieve statistical significance.

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metabolism of all classes of macronutrients. This indicates a concurrent impact of diabetes on intermediary patterns in both PC dimensions (see Figure 4 legend). Those of others [42;43;46] is a higher concentration of 2-HB than others. One common finding comparing our results to others is a higher concentration of 2-HB in T2DM. Increased in Diabetes:

- 3-hydroxybutyric acid (3-HB) 10676 ± 1455
- oleic acid 8837 ± 1105
- glycerol phosphate 23377 ± 2189
- fructose 25503 ± 34001
- palmitoleic acid 6286 ± 1444
- 3,6-anhydroglucitol 1660 ± 147
- glucuronic acid 1718 ± 151
- glucose 105732 ± 90953
- heptadecenoic acid 11630 ± 554
- iminodiacetate 1009 ± 106
- leucine 110271 ± 14147
- 2-hydroxybutyric acid (2-HB; \(\alpha\)-ketobutyrate) 100560 ± 21376
- 2-deoxyerythritol 8270 ± 727
- palmitic acid 75185 ± 5308
- 2-ketoisocaproic acid (\(\alpha\)-ketoisocaproate) 4809 ± 462
- uridine 850 ± 51
- cystine 30534 ± 3583
- xylose 4388 ± 290
- histidine 44969 ± 2332
- stearic acid 598153 ± 31201

Values are quantifier peak height means ± SEM; see Supplemental Table S2 for information on unknown metabolites significantly changed in T2DM.

| Metabolite | Non-Diabetic (n = 43) | Diabetic (n = 43) | Relevant Metabolic Pathway | Diabetic/Non-Diabetic Ratio |
|------------|----------------------|------------------|---------------------------|---------------------------|
| Increased in Diabetes: |                     |                  |                           |                           |
| 3-hydroxybutyric acid (3-HB) | 10676 ± 1455 | 47424 ± 9450 | lipid/fatty acid | 4.44* |
| oleic acid | 8837 ± 1105 | 23377 ± 2189 | lipid/fatty acid | 2.65*** |
| glycerol phosphate | 23377 ± 2189 | 11400 ± 1018 | lipid/fatty acid | 1.81* |
| fructose | 25503 ± 34001 | 517922 ± 18549 | carbohydrate | 2.03**** |
| palmitoleic acid | 6286 ± 1444 | 11400 ± 1018 | lipid/fatty acid | 1.81* |
| 3,6-anhydroglucitol | 1660 ± 147 | 2920 ± 116 | carbohydrate (microbial?) | 1.76**** |
| glucuronic acid | 1718 ± 151 | 2844 ± 264 | carbohydrate | 1.66* |
| glucose | 105732 ± 90953 | 1644213 ± 56650 | carbohydrate | 1.56**** |
| heptadecenoic acid | 11630 ± 554 | 17911 ± 1564 | lipid/fatty acid | 1.54* |
| iminodiacetate | 1009 ± 106 | 1546 ± 92 | carbohydrate | 1.53** |
| leucine | 110271 ± 14147 | 164281 ± 9806 | amino acid | 1.49** |
| 2-hydroxybutyric acid (2-HB; \(\alpha\)-ketobutyrate) | 100560 ± 21376 | 146853 ± 9844 | amino acid | 1.46* |
| 2-deoxyerythritol | 8270 ± 727 | 10950 ± 383 | lipid/fatty alcohol | 1.32** |
| palmitic acid | 75185 ± 5308 | 98294 ± 6003 | lipid/fatty acid | 1.31* |
| 2-ketoisocaproic acid (\(\alpha\)-ketoisocaproate) | 4809 ± 462 | 6169 ± 309 | amino acid | 1.28* |
| uridine | 850 ± 51 | 1085 ± 51 | pyrimidine | 1.28* |
| cystine | 30534 ± 3583 | 38496 ± 1818 | amino acid | 1.26* |
| xylose | 4388 ± 290 | 5479 ± 221 | carbohydrate/pentose phosphate | 1.25* |
| histidine | 44969 ± 2332 | 56071 ± 2178 | amino acid | 1.25** |
| stearic acid | 598153 ± 31201 | 719217 ± 24664 | lipid/fatty acid | 1.20** |

Decreased in Diabetes:

- benzyl alcohol | 17762 ± 1062 | 15741 ± 405 | phenolic metabolite or xylene (microbial?) | 0.89* |
- benzoic acid | 37841 ± 2445 | 32968 ± 1066 | phenolic metabolite or xylene (microbial?) | 0.88* |
- lysine | 170439 ± 13635 | 141626 ± 6008 | amino acid | 0.83* |
- ethanolamine | 479789 ± 42252 | 380214 ± 19511 | choline precursor | 0.79* |
- arachidonic acid | 35123 ± 3669 | 26058 ± 1410 | lipid/fatty acid | 0.74** |
- glycine | 326074 ± 41720 | 239650 ± 16035 | amino acid | 0.74* |
- glycerol-3-phosphate | 23920 ± 2430 | 16571 ± 1018 | glycerophospholipid | 0.69*** |

Values are quantifier peak height means ± SEM; see Supplemental Table S2 for information on unknown metabolites significantly changed in T2DM.

**p < 0.05; ***p < 0.001; ****p < 0.0001 (unpaired t-test). doi:10.1371/journal.pone.0015234.t002

Further supporting that insulin resistance and T2DM are conditions associated with abnormal amino acid metabolism, elevated blood concentrations of BCAAs have been consistently observed in rodent models of obesity [5–9;48] and in obese and/or...
Figure 1. Relative plasma concentrations of leucine (A) and valine (B) are increased in type 2 diabetic African-American obese women. Results are expressed as total quantifier peak height percent of all summed plasma amino acid (AA) peak heights, i.e., as % of total AA. Bars represent the mean ± SEM for n = 12 and n = 43 non-diabetic and diabetic subjects, respectively. **p < 0.01, unpaired t-test. Absolute quantifier peak heights are presented in the Results. doi:10.1371/journal.pone.0015234.g001

Figure 2. Plasma branched chain amino acid (BCAA) concentrations are correlated with fasting HbA1c% and plasma acylcarnitine concentrations in non-obese (yellow circles) and type 2 diabetic (blue circles) obese women. Shown are correlations between plasma leucine (A,C) and valine (B,D) enrichments (% of their concentrations relative to total measured amino acid concentrations; see Figure 1 legend) with fasting blood HbA1c (top panels) or plasma acetylcarnitine concentration (bottom panels). Pearson’s r and p values for the correlations are given within the figures. doi:10.1371/journal.pone.0015234.g002
T2DM human subjects (i.e., [8,49]). In line with these findings, plasma leucine, 2-ketoisocaproic acid, and valine concentrations and/or their relative abundance in the total AA pool were increased in obese type 2 diabetic vs. obese non-diabetic African-American women in the current study (Figure 2). It has been asserted that higher concentrations of BCAA in obesity cause or exacerbate insulin resistance through mechanisms involving activation of the molecular target of rapamycin (mTOR)[8]. However, this model remains controversial. First, it is not clear that the magnitude of increase in fasting blood BCAA in obesity or T2DM are of high enough magnitude to trigger mTOR to a level that would negatively impact insulin action in situ. Second, BCAA-rich dairy-based diets have consistently been shown to have anti-obesity properties in rodent models (see [50]). Third, leucine supplementation to diet-induced obese mice either had no effect [51] or substantially improved [52] metabolic profiles. Finally, protein-rich diets often have positive metabolic effects in type 2 diabetic and obese humans (see [53;54]).

Thus, we favor the idea that higher fasting blood BCAA (and some other AA) in insulin-resistant states results simply marks reduced catabolism in key tissues, and this reduced catabolism is hypothesized to limit tissue concentrations of AA derivatives important to normal metabolism. Supporting this perspective are reports indicating that the two initial catabolic enzymes of BCAA catabolism, mitochondrial branched-chain amino acid aminotransferase (BCATm) and branched-chain a-ketoacid dehydrogenase (BCKD) expression and specific activities are reduced in the liver and/or white adipose tissue (WAT) of obese rodents [6;7;9;48]. She et al. [6] demonstrated that obese human subjects who underwent bariatric surgery had significantly reduced plasma BCAA concentration 1 yr post-surgery concurrent with increased WAT BCKD and BCATm activities. Whole-body BCAA clearance was reportedly ~20% reduced in T2DM subjects [4] and recently an insulin resistance effect on leucine protein fractional synthesis rate was demonstrated [55;56]. However, other reports have indicated that leucine oxidation or amino acid turnover is not impaired in obese or type 2 diabetic persons [57–63]. Clearly, additional research is needed to identify tissue-specific alterations in the fates of protein and amino acids in obesity and diabetes to better understand the basis for elevations in blood concentrations of BCAA and certain other amino acids under these conditions. Interestingly, higher circulating concentrations of 2-HB are associated with perturbation in biotin metabolism, specifically in inherited biotinidase deficiencies [64], and poor biotin status has been associated with insulin resistant

Figure 3. Relative plasma concentrations of propionylcarnitine (green symbols) were reduced concurrent with increases in the relative plasma concentrations of a precursor, valine, with increasing plasma acetyl carnitine concentration in obese African-American women. Symbols represent individuals included in metabolomics studies described in the text. doi:10.1371/journal.pone.0015234.g003

Figure 4. Separation of non-diabetics (yellow circles) from type 2 diabetics (blue circles) due to variance in plasma metabolite factors. Principal components analysis (PCA) in dimensions 1 and 2 using 158 metabolites illustrates differential distribution of diabetic and non-diabetic subjects along the PC1 (X axis) and PC2 (Y axis) axes, with each symbol plotting PC1-PC2 scores for a given subject. Metabolite components whose variance-derived loadings values contributed most to the PC separations scores are listed in Supplemental Table S3. Summarizing the loading contributions from known compounds, elevated fatty acids and the enrichment of the amino acid pool with branched chain AAs segregated diabetics from controls in PC1, while elevations in various carbohydrates as well as a suite of amino acids separated diabetics from controls in PC2. doi:10.1371/journal.pone.0015234.g004
associated with insulin resistance, since valine, isoleucine, and cysteine are precursors to succinate and succinyl-CoA. Consistent with this view, plasma valine enrichment in the amino acid pool rose concurrent with reductions in relative concentrations of propionylcarnitine (a proxy for a valine anaplerotic product, propionyl-CoA) as blood sugar control worsened and plasma acetyl carnitine accumulation became more apparent in our study cohort (Figure 5). In theory, changes in matrix α-ketoglutarate concentration (i.e., via conversion to glutamate or export via the α-ketoglutarate carrier) could impact both TCA cycle function and BCAM metabolism, since BCATm activity involves α-ketoglutarate. Notably, Seifert et al. recently reported that increases in the rate of LCFA combustion and concurrent incomplete LCFA β-oxidation are associated with muscle mitochondrial export of α-ketoglutarate [71], providing a potential connection between mitochondrial lipid metabolism and control of α-ketoglutarate export. The idea that anaplerotic stress and anaplerotic/cataleptic balance are factors underlying or exacerbating metabolic dysfunction in T2DM is compelling, but clearly requires experimental validation.

Our work has been driven in part by a desire to identify muscle-specific metabolites associated with LCFA β-oxidation, considering the importance of efficient skeletal muscle LCFA catabolism to maintenance of insulin sensitivity. An interesting group to study in this regard are non-diabetic obese African-American persons harboring a G304A (g/a) missense allele leading to truncated UCP3, and who have been found to have significantly reduced whole-body lipid oxidation [27]. This must in large part emanate from muscle considering the almost exclusive expression of this mitochondrial carrier in that tissue in humans [72]. Thus, differences in circulating metabolites when comparing g/g to g/a individuals may reflect differences in muscle metabolism. In this study, we found several metabolites that were altered in overnight-fasted non-diabetic g/a allele carriers (Table 1, Table S1), and most await identification and to ascertain tissue-specificity of their production and utilization. Many metabolite differences between genotypes were statistically significant, but significance was not detected after application of a 20% false discovery rate. This was likely due to limited sample sizes, and indicates that additional comparative studies are warranted using larger cohorts of g/g and g/a polymorphic subjects to confirm metabolite differences observed herein. We observed reductions in plasma concentrations of α-ketoglutarate and glutamate in g/a individuals, as well as lower cysteine, suggesting that alteration in UCP3 function in muscle of obese African-American women impacts TCAi dynamics and amino acid metabolism. Plasma butyrylcarnitine (C4-carnitine) was previously found to be reduced by 57% in the same non-diabetic g/a subjects [23]. Plasma butyrylcarnitine should be reflective of tissue butyryl-CoA concentrations, and importantly the latter is a common metabolite in the catabolism of both glutamate and α-ketoglutarate. Thus, we propose from the aggregate of results that muscle UCP3 activity may somehow play a role in regulating tissue α-ketoglutarate/glutamate dynamics.

In summary, broad metabolite profiling of non-diabetic vs. type 2 diabetic plasma, in conjunction with our previous acylcarnitine profiling, indicates that T2DM is a disease that disrupts multiple intermediary metabolic pathways including amino acid metabolism. Our data support a hypothetical working model in which attenuated BCAA, and possibly cysteine, catabolism contribute to increased blood concentrations of these amino acids and their derivatives in the insulin-resistant state, and we speculate that this contributes to anaplerotic stress that is associated with incomplete oxidation of LCFA and accumulation of acylcarnitines in T2DM. Variation in a subset of metabolites, including 2-HB, discriminated
T2DM from non-diabetics and strongly correlated with HbA1c. These metabolites therefore are of interest as potential biomarkers of disease status and glucose homeostasis. Our results also point to, for the first time, a potential role of mitochondrial UCP3 in regulation of the glutamate/glutathione/ubiquinol-CoA tissue pool.

Supporting Information

Table S1 Plasma Metabolite list comparing UCP3 g/g and g/a polymorphs in an obese, African-American cohort of women. Values are quantifier peak heights determined for each subject used in the analyses, and metabolites are identified in the leftward column. Mass spec information is provided in columns B–E, and in some cases, KEGG identifiers are provided. Unpaired t-tests were used to evaluate non-diabetic genotype differences, as noted.

Table S2 Plasma Metabolite list comparing type 2 diabetic and non-diabetic obese African-American women. Values are quantifier peak heights determined for each subject used in the analyses, and metabolites are identified in the leftward column. Mass spec information is provided in columns B–E, and in some cases, KEGG identifiers are provided. Unpaired t-tests were used to evaluate differences between diabetic and non-diabetic women, as noted.

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Author Contributions

Conceived and designed the experiments: SHA. Performed the experiments: OF WTG KHL. Analyzed the data: SHA OF JWN WTG. Contributed reagents/materials/analysis tools: OF JWN WTG KHL CLH. Wrote the paper: SHA.

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