

Metabolite Profiles During Oral Glucose Challenge

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To identify distinct biological pathways of glucose metabolism, we conducted a systematic evaluation of biochemical changes after an oral glucose tolerance test (OGTT) in a community-based population. Metabolic profiling was performed on 377 nondiabetic Framingham Offspring cohort participants (mean age 57 years, 42% women, BMI 30 kg/m²) before and after OGTT. Changes in metabolite levels were evaluated with paired Student *t* tests, cluster-based analyses, and multivariable linear regression to examine differences associated with insulin resistance. Of 110 metabolites tested, 91 significantly changed with OGTT ($P \leq 0.0005$ for all). Amino acids, β -hydroxybutyrate, and tricarboxylic acid cycle intermediates decreased after OGTT, and glycolysis products increased, consistent with physiological insulin actions. Other pathways affected by OGTT included decreases in serotonin derivatives, urea cycle metabolites, and B vitamins. We also observed an increase in conjugated, and a decrease in unconjugated, bile acids. Changes in β -hydroxybutyrate, isoleucine, lactate, and pyridoxate were blunted in those with insulin resistance. Our findings demonstrate changes in 91 metabolites representing distinct biological pathways that are perturbed in response to an OGTT. We also identify metabolite responses that distinguish individuals with and without insulin resistance. These findings suggest that unique metabolic phenotypes can be unmasked by OGTT in the prediabetic state. *Diabetes* 62:2689–2698, 2013

Diabetes affects >1 in 10 adults 20 years of age or older in the U.S., and more than one-third of all adults have prediabetes (1). Changes in traditional measures of glucose and insulin metabolism are known to occur years before the diagnosis of diabetes is made (2). Using high-throughput profiling of metabolic status, we have shown that elevations in plasma

branched-chain and aromatic amino acids are also able to predict future diabetes in otherwise normoglycemic, healthy adults (3). Similarly, lipid profiling has demonstrated novel perturbations in triacylglycerol distribution that signal future diabetes risk (4). These findings highlight how emerging technologies are able to broaden our perspective on early disease states, potentially lending insights into biological mechanisms that underlie diabetes and metabolic disease. Characterizing early metabolic changes may also lead to the early identification of at-risk individuals and may prompt the initiation of proven preventive strategies (5).

The oral glucose tolerance test (OGTT) provides a dynamic view of glucose and insulin physiology and has been widely used for decades to diagnose diabetes (6,7). Therefore, we conducted a systematic evaluation of biochemical changes after OGTT in a community-based population, with the goal of providing a broad view of the metabolic response to a glucose challenge. An important advantage of profiling plasma samples before and after glucose ingestion is that each individual is able to serve as their own biological control. In addition to attenuating noise attributable to interindividual variation, this approach limits confounding effects of diet, medications, and other inputs that impact the human metabolome. We used a liquid chromatography/mass spectrometry (LC/MS)-based platform that allowed highly specific identification of small molecules in a targeted manner. In prior pilot studies, our group has shown that metabolite excursions with OGTT revealed a switch from catabolism to anabolism, largely attributable to insulin actions (8). In the current study, we sought to evaluate perturbations with OGTT in an expanded panel of metabolites and in a more comprehensive population-based sample with a high propensity for the development of diabetes, and to investigate these changes in individuals with and without insulin resistance.

RESEARCH DESIGN AND METHODS

Study sample. The Framingham Heart Study (FHS) Offspring cohort is a prospective, observational, community-based cohort (9). The children (and their spouses) of FHS participants were recruited in 1971 and have been followed with serial examinations approximately every 4 years. Sample selection procedures have previously been described (3). In brief, study participants included in this analysis attended the fifth examination (1995–1998), and they underwent metabolic profiling on pre- and post-glucose challenge samples. A total of 377 individuals free of diabetes were included in this analysis. Participants provided informed consent, and the study protocol was approved by the institutional review board at Boston University Medical Center.

Clinical assessment. Subjects underwent a 2-h, 75-g OGTT after a 12-h overnight fast. Blood samples were obtained before and after OGTT. Diabetes was defined as a fasting glucose ≥ 126 mg/dL, nonfasting blood glucose ≥ 200 mg/dL, or the use of insulin or other hypoglycemic medications. Hypertension was defined as a systolic blood pressure (SBP) ≥ 140 mmHg, a diastolic blood pressure ≥ 90 mmHg, or current use of medication to treat hypertension. Participants smoking cigarettes regularly during the prior year were considered current smokers. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting insulin (μ IU/mL) \times fasting glucose

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See accompanying commentary, p. 2651.

(mmol/mL)/22.5 (10) and insulin resistance defined as the top quartile of HOMA-IR from the entire FHS Offspring cohort free of diabetes at the fifth examination cycle. Although individuals were not selected based on insulin resistance status, the sample was selected based on a high propensity for the future development of diabetes as previously described (3).

Metabolic syndrome was defined as the presence of any three of the following five criteria (11): waist circumference ≥ 40 inches in men or ≥ 35 inches in women, triglycerides ≥ 150 mg/dL or treatment with a fibrate or niacin, HDL cholesterol < 40 mg/dL in men or < 50 mg/dL in women or treatment with a fibrate or niacin, SBP ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg or drug treatment for hypertension, or fasting glucose ≥ 100 mg/dL or drug treatment for elevated glucose.

Metabolic profiling. Blood samples were collected after an overnight fast, both immediately prior to and 2 h after administration of a standard 75-g OGTT. Samples were immediately centrifuged and stored at -80°C until assayed. The details of the LC/MS platform used to profile analytes have been previously described (3). In brief, data were acquired using a 4000 QTRAP triple quadrupole mass spectrometer (Applied Biosystems/Sciex, Foster City, CA) and a multiplexed LC system consisting of two 1200 Series pumps (Agilent Technologies) and an HTS PAL autosampler (Leap Technologies). Polar plasma metabolites were measured using hydrophilic interaction chromatography and tandem MS with electrospray ionization and multiple reaction monitoring scans in the positive ion mode as previously detailed (3). We also performed a complementary analysis of small molecules preferentially ionized using negative mode electrospray ionization under basic conditions and used hydrophilic interaction chromatography to assess organic acids and other intermediary metabolites. Formic acid, ammonium acetate, and LC/MS-grade solvents (Sigma-Aldrich) were used, and samples were prepared by adding 120 μL of extraction solution (80% methanol plus the internal standards inosine- $^{15}\text{N}_4$, thymine- d_4 , and glycocholate- d_4) to 30 μL of plasma. The samples were centrifuged (10 min, 10,000 rpm, 4°C) and supernatants were injected directly. For quality control, internal standard peak areas were monitored and individual samples with peak areas differing by > 2 SD from the group mean were reanalyzed. For this analysis, 54% of the metabolites had a coefficient of variation $\leq 10\%$, and 74% had a coefficient of variation $\leq 20\%$.

Statistical analysis. Baseline characteristics were summarized by insulin resistance status. Metabolites with $> 20\%$ missing or undetectable values were excluded; 110 metabolites were analyzed. Metabolite levels were log transformed due to right-skewed distribution, and paired Student *t* tests were conducted to examine the differences between post- and pre-OGTT metabolite levels. To account for multiple testing, a Bonferroni-corrected threshold, $P < 0.05/110 = 0.0005$, was used to define a statistically significant change in metabolite levels with glucose challenge. Pearson correlation coefficients were calculated to identify groupings of metabolites.

Metabolites with a > 1.2 -fold change (based on the ratio of the log-transformed metabolites) after OGTT were selected to test for differences between subsets with and without insulin resistance. To minimize multiple hypothesis testing, metabolites within the same grouping were only selected if there was low-moderate correlation between metabolites (defined as absolute $r < 0.4$). Multivariable linear regression was used to test differences in OGTT metabolite changes based on HOMA-IR and between groups with and without insulin resistance; models were first adjusted for age and sex and further adjusted for BMI. Results were deemed significant at a Bonferroni-corrected threshold, $P < 0.05/27 = 0.002$. Least squares-adjusted mean differences were graphed. To explore whether differences in metabolite excursion in insulin-resistant individuals were potentially mediated by insulin effects, we examined the relationship of insulin excursion during OGTT and the corresponding metabolite excursion using multivariable linear regression.

In secondary analyses, metabolites with significant changes during glucose challenge were then included in a cluster-based analysis. The change in metabolite with OGTT (Δ) was defined as the log-post-OGTT metabolite level minus the log-pre-OGTT metabolite level. The Δ in metabolite levels with OGTT were rank normalized using the Bloomberg method, and correlated Δ were classified into hierarchical groupings using cluster analysis (PROC VARCLUS). Clusters were formed on the basis of principal components, using eigenvalue thresholds of 1.

A cluster score was then calculated for each participant, based on the average of the rank-normalized Δ for metabolites within each cluster. Multivariable regression models were used to examine the association of cluster scores and glycemic traits, including insulin resistance, defined as the upper quartile of HOMA-IR in the nondiabetic participants of the Framingham Offspring cohort who attended exam 5; impaired glucose tolerance, defined as a 2-h post-OGTT glucose of 140–199 mg/dL; and impaired fasting glucose, defined as a fasting glucose 100–125 mg/dL. Models were first adjusted for age and sex and further adjusted for BMI. The study sampling scheme was accounted for in PROC GLIMMIX. For clusters with significant Bonferroni-adjusted *P* values, the same regression models were performed on individual

metabolite Δ to examine the association of individual metabolites with each glycemic trait.

All analyses were conducted using SAS 9.3 (Cary, NC), and PROC GLIMMIX was used to account for the sampling scheme.

RESULTS

A total of 377 subjects (mean age 57 years, 42% women) were included in this analysis. Due to the selection of a sample with a high propensity for the development of diabetes, half of the sample met the definition of insulin resistance, with HOMA-IR in the top quartile of the full nondiabetic FHS Offspring sample. Insulin-resistant individuals tended to have a higher prevalence of metabolic syndrome or features of the metabolic syndrome (Table 1).

Insulin levels increased by 65 ± 55 $\mu\text{IU/mL}$ ($P < 0.0001$) 2 h after glucose challenge, and glucose levels increased by 17 ± 30 mg/dL ($P < 0.0001$) at 2 h post-OGTT in the overall sample. Insulin and glucose excursion with OGTT were positively correlated ($r = 0.53$, $P < 0.0001$). Participants with insulin resistance had higher baseline insulin levels, with higher insulin and glucose excursion after OGTT, compared with those who were not insulin resistant (insulin increased by 89 ± 63 vs. 41 ± 31 $\mu\text{IU/mL}$, $P < 0.0001$; glucose increased by 24 ± 29 vs. 10 ± 29 mg/dL, $P < 0.0001$).

Of the 110 metabolites that were detectable in at least 80% of the sample, 91 significantly increased or decreased in level after the glucose challenge ($P \leq 0.0005$ for all). The majority of metabolites decreased in response to OGTT (Table 2), whereas a few increased in response to glucose challenge (Table 3). The largest relative decreases were seen in β -hydroxybutyrate, which decreased by more than fourfold after glucose challenge, and serotonin, which decreased more than twofold. By contrast, the largest increase was in hippurate (a known derivative of the preservative benzoic acid found in glucola, the drink used during OGTT) (12), which increased more than fourfold, and phosphoglycerate, which increased by $\sim 50\%$. Older

TABLE 1
Characteristics of sample by insulin resistance status

	Insulin resistance status		
	Total (n = 377)	Normal (n = 189)	Resistant (n = 188)
Clinical characteristics			
Age (years)	56.5 (8.8)	56.3 (8.8)	56.7 (8.8)
Women, %	42	44	40
BMI (kg/m^2)	30.2 (5.3)	28.4 (4.6)	32.0 (5.3)
Waist circumference (cm)	101 (13)	97 (12)	105 (12)
SBP (mmHg)	133 (18)	132 (19)	134 (17)
Antihypertensive treatment, %			
	30	25	35
Current smoker, %	16	17	16
Metabolic syndrome, %	67	51	82
Laboratory characteristics			
Fasting glucose (mg/dL)	105 (9)	103 (9)	106 (8)
2-h glucose (OGTT) (mg/dL)	122 (31)	113 (30)	131 (29)
Fasting insulin ($\mu\text{IU/mL}$)	12.8 (9.4)	6.4 (2.8)	19.2 (9.4)
HOMA-IR	3.3 (2.5)	1.6 (0.7)	5.0 (2.5)
Total cholesterol (mg/dL)	211 (36)	213 (39)	208 (33)
HDL cholesterol (mg/dL)	45 (13)	49 (14)	41 (11)
Triglycerides (mg/dL)	172 (105)	144 (86)	200 (114)

Data are presented as mean (SD) unless otherwise indicated.

TABLE 2
Metabolites that decreased after glucose challenge (paired Student *t* test, *P* < 0.0001 for all)

Metabolite	Pathway group	Mean change*	SD	Fold change
β-Hydroxybutyrate	Ketone	-1.51	0.72	4.51
Serotonin	Tryptophan derivative	-0.85	0.83	2.33
Hypoxanthine	Purine	-0.78	0.93	2.17
Xanthine	Purine	-0.52	0.57	1.68
Citrulline	Urea cycle	-0.50	0.15	1.65
Guanosine diphosphate	Purine	-0.49	0.72	1.63
Isoleucine	Amino acid	-0.47	0.14	1.60
Leucine	Amino acid	-0.45	0.15	1.57
Inosine	Purine	-0.40	0.49	1.49
Xanthurenate	Amino acid metabolite	-0.39	0.31	1.47
Propionate	Organic acid	-0.38	0.42	1.46
Guanosine monophosphate	Purine	-0.37	0.66	1.45
Methionine	Amino acid	-0.36	0.19	1.44
Gentisate	Amino acid metabolite	-0.34	0.47	1.41
Tyrosine	Amino acid	-0.34	0.14	1.41
Cholate	Bile acid	-0.33	0.82	1.40
Adenosine monophosphate	Purine	-0.33	0.76	1.39
Hydroxyproline	Amino acid	-0.31	0.15	1.37
Asparagine	Amino acid	-0.31	0.24	1.36
Adenosine diphosphate	Purine	-0.29	0.84	1.33
Salicylurate		-0.28	0.46	1.33
Uridine diphosphate	Pyrimidine	-0.27	0.43	1.31
Niacinamide		-0.27	0.40	1.31
Threonine	Amino acid	-0.27	0.11	1.31
Aspartate	Amino acid	-0.27	0.30	1.31
Phenylalanine	Amino acid	-0.26	0.14	1.30
Ornithine	Urea cycle	-0.25	0.25	1.29
Amino adipate	Amino acid metabolite	-0.25	0.23	1.28
Serine	Amino acid	-0.24	0.17	1.27
Orotate	Vitamin	-0.24	0.28	1.27
Valine	Amino acid	-0.23	0.10	1.26
Uridine monophosphate	Pyrimidine	-0.23	0.46	1.25
Cystathionine	Amino acid metabolite	-0.22	0.32	1.25
Arginine	Amino acid	-0.22	0.35	1.24
Pyridoxate	Vitamin	-0.20	0.16	1.22
Deoxycholates	Bile acid	-0.19	0.41	1.21
Xanthosine		-0.17	0.25	1.19
Lysine	Amino acid	-0.17	0.14	1.18
Histidine	Amino acid	-0.17	0.14	1.18
Tryptophan	Amino acid	-0.16	0.14	1.17
α-Hydroxybutyrate	Organic acid	-0.16	0.17	1.17
ADMA/SDMA		-0.15	0.15	1.16
Ribose/ribulose phosphate	Sugar	-0.15	0.40	1.16
Thiamine		-0.15	0.70	1.16
5-HIAA	Tryptophan derivative	-0.14	0.39	1.15
Glycine	Amino acid	-0.14	0.17	1.15
Glutamate	Amino acid	-0.14	0.21	1.15
NMMA		-0.14	0.18	1.15
Proline	Amino acid	-0.14	0.09	1.15
Taurine		-0.14	0.12	1.14
Hydroxyphenylacetate	Amino acid metabolite	-0.13	0.19	1.13
Indole propionate	Amino acid metabolite	-0.12	0.25	1.13
Oxalate	Dicarboxylic acid	-0.12	0.21	1.12
Fumarate/maleate	TCA cycle	-0.11	0.22	1.12
Flavin mononucleotide	Vitamin	-0.11	0.44	1.12
Aconitate	TCA cycle	-0.10	0.21	1.11
Glutamine	Amino acid	-0.10	0.26	1.10
Pantothenate	Vitamin	-0.09	0.15	1.10
Uridine	Pyrimidine	-0.09	0.16	1.10
Trimethylamine		-0.09	0.24	1.09
Isocitrate	TCA cycle	-0.08	0.18	1.08
Methyladipate/pimelate	Dicarboxylic acid	-0.07	0.18	1.07
Glucuronate	Sugar	-0.07	0.23	1.07

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TABLE 2
Continued

Metabolite	Pathway group	Mean change*	SD	Fold change
Alanine	Amino acid	-0.07	0.13	1.07
Sorbitol	Sugar	-0.07	0.19	1.07
Quinolinic acid	Amino acid metabolite	-0.07	0.16	1.07
Hydroxyglutarate	Organic acid	-0.06	0.25	1.07
Dimethylglycine	Methyl	-0.05	0.12	1.06
F1P/F6P/G1P/G6P	Glycolysis	-0.05	0.28	1.05
Adipate	Dicarboxylic acid	-0.05	0.23	1.05
Suberate	Dicarboxylic acid	-0.05	0.19	1.05
Sebacate	Dicarboxylic acid	-0.04	0.19	1.04
Urate	Purine	-0.03	0.10	1.03

ADMA, asymmetric dimethylarginine; F1P, fructose 1-phosphate; F6P, fructose 6-phosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; NMMA, *N*-monomethylarginine; SDMA, symmetric dimethylarginine. *Mean change represents log-post-OGTT value minus log-pre-OGTT value.

age was associated with greater increases in glucose/fructose/lactose with OGTT ($P = 0.0003$). After adjustment for age and baseline metabolite levels, women had greater decreases in citrulline and xanthosine and greater increases in bile acids, phosphoenolpyruvate, and glucose/fructose/lactose with glucose challenge when compared with men ($P < 0.0005$ for all) (Supplementary Table 1).

Correlated metabolites reflect biological pathways. In analyses using a cluster-based approach, we found that groupings of metabolites whose baseline concentrations correlated, or whose responses to glucose challenge correlated, also reflected common underlying biological groupings. For example, baseline amino acid concentrations, as well as their responses to OGTT, were highly correlated with each other (Supplementary Tables 2 and 3 and Fig. 1). **Insulin actions on known metabolic pathways.** Changes in metabolite levels in response to glucose challenge appeared to be consistent across metabolic pathways (Fig. 2), highlighting the multiple physiological actions of insulin. Decreases in all amino acids and related metabolites provided evidence of decreased proteolysis. Similarly, a decrease in β -hydroxybutyrate and tricarboxylic acid (TCA)

cycle intermediates was consistent with inhibition of ketogenesis and TCA cycle flux. In contrast, products of glycolysis increased after glucose challenge. Our findings also show a decrease in nitrogenous bases, including purines and pyrimidines, in response to glucose challenge, consistent with insulin-mediated stimulation of DNA and purine biosynthesis across different tissues as seen in previous experimental studies (13,14).

Insulin actions on novel biological pathways. We found consistent changes in several biological pathways that are less established in relation to glucose and insulin metabolism (Fig. 3). Serotonin and its precursors/derivatives, including 5-hydroxyindoleacetic acid (5-HIAA) and tryptophan, all decreased after glucose challenge. Metabolites of the urea cycle, including citrulline, ornithine, and arginine, all decreased. Nucleic acids and related metabolites decreased as well in response to OGTT. We also observed decreases in a number of derivatives or cofactors related to the B vitamins, a group of water-soluble vitamins essential to cellular metabolism: niacinamide, the amide of nicotinic acid (vitamin B3/niacin), is important in various oxidation-reduction reactions; pyridoxate, an enzyme in the family of

TABLE 3
Metabolites that increased after glucose challenge (paired Student *t* test, $P < 0.0001$ for all)

Metabolite	Group	Mean change*	SD	Fold change
Hippurate	Amino acid metabolite	1.53	0.80	4.63
Phosphoglycerate	Glycolysis	0.39	0.61	1.48
Taurodeoxycholates	Bile acid	0.34	0.86	1.41
Glycodeoxycholates	Bile acid	0.30	0.82	1.35
α -Glycerophosphate	Glycolysis	0.30	0.49	1.35
Taurocholate	Bile acid	0.24	0.84	1.27
Glycocholate	Bile acid	0.15	0.63	1.17
Glycerophosphocholine	Methyl	0.14	0.38	1.16
Lactate	Glycolysis	0.13	0.22	1.14
Phosphoenolpyruvic acid	Glycolysis	0.10	0.33	1.11
Phosphocreatine		0.10	0.32	1.10
Glucose/fructose/lactose	Sugar	0.06	0.24	1.06
Inositol	Sugar	0.06	0.20	1.06
Creatine		0.05	0.14	1.05
Fructose/glucose/galactose	Sugar	0.04	0.14	1.05
Carnitine		0.03	0.08	1.03
Choline	Methyl	0.03	0.12	1.03
Betaine	Methyl	0.03	0.08	1.03

*Mean change represents log-post-OGTT value minus log-pre-OGTT value.

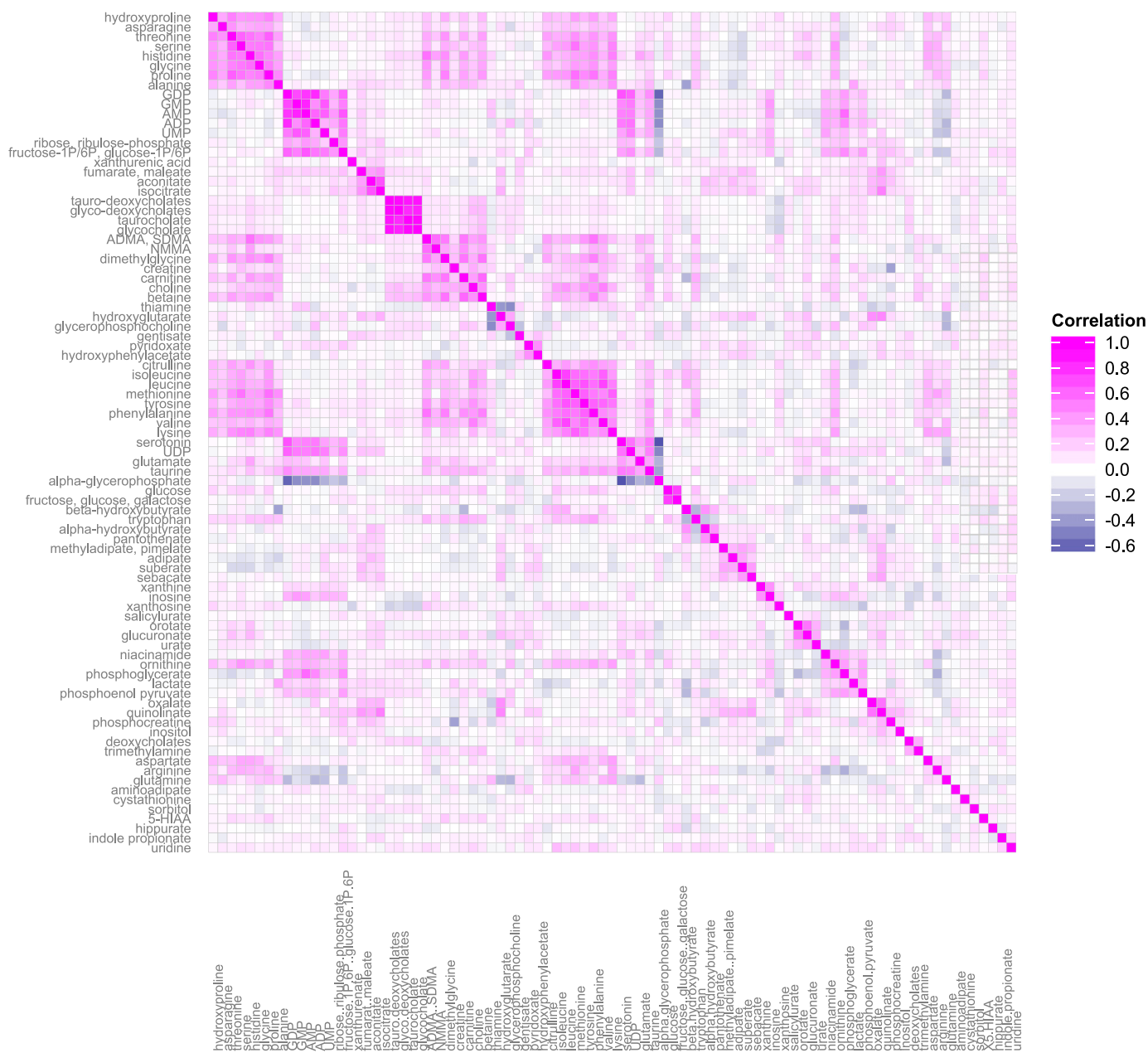


FIG. 1. Heat map of correlated metabolite changes with glucose challenge.

oxidoreductases, plays a crucial role in vitamin B6 metabolism; thiamine (vitamin B1) is a coenzyme in glucose and amino acid metabolism; flavin mononucleotide is a derivative of riboflavin (vitamin B2) and serves as a cofactor for a various oxidoreductases; and pantothenate (vitamin B5) is an essential cofactor for CoA synthesis and metabolism of proteins, carbohydrates, and fats. Lastly, we observed an increase in conjugated bile acids and a concomitant decrease in unconjugated bile acids in response to the glucose challenge.

Effect of insulin resistance and other glycemic traits on metabolite excursions. A total of 27 uncorrelated ($r < 0.4$) metabolites had a >1.2 -fold change after OGTT. For five of these metabolites, the excursion after OGTT was different in participants with normal insulin levels compared with insulin-resistant individuals, including β -hydroxybutyrate, isoleucine, lactate, orotate, and pyridoxate

(Table 4). The excursion of all metabolites except orotate was blunted in insulin-resistant, compared with noninsulin-resistant, individuals (Fig. 4). Of these metabolites, changes in lactate, but not the other metabolites, were associated with insulin excursion during OGTT in the overall sample in exploratory analyses ($P < 0.0001$ after adjustment for age, sex, and insulin resistance status) and remained associated after exclusion of insulin-resistant individuals ($P = 0.0005$). When examined as a continuous variable, additional suggestive differences were observed according to HOMA-IR in taurodeoxycholates, niacinamide, and ornithine, with a blunted response in those with greater insulin resistance (Supplementary Table 4).

In secondary analyses using a cluster-based approach, changes in hydroxyphenylacetate and phosphoenolpyruvate with OGTT had suggestive associations with fasting glucose. Glucose/fructose/lactose, fructose/glucose/galactose,

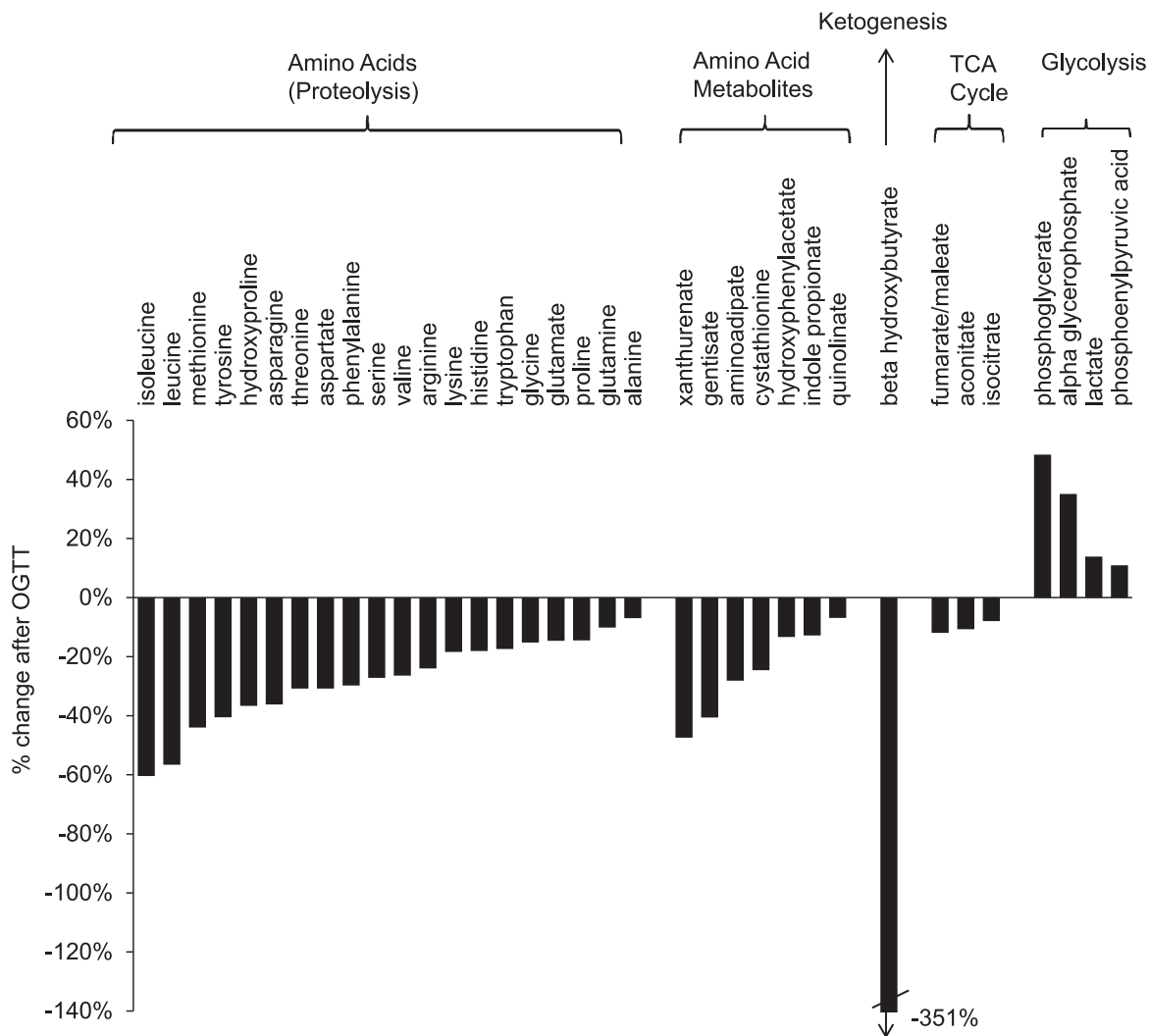


FIG. 2. Metabolite excursions after glucose challenge in metabolic pathways that reflect the effects of insulin. Notable perturbations include decreased proteolysis (as evidenced by lower amino acids and amino acid metabolites), inhibition of ketogenesis (lower β -hydroxybutyrate), suppression of the TCA cycle, and activation of glycolysis. % change = $100 \times (\text{up-fold change} - 1)$ or $100 \times (1 - \text{down-fold change})$.

and hippurate had significant associations with 2-h post-OGTT glucose, and β -hydroxybutyrate, tryptophan, α -hydroxybutyrate, and ornithine had suggestive associations (Supplementary Table 5).

DISCUSSION

Using a systematic approach, we have demonstrated perturbations in numerous metabolic and biological pathways in response to a glucose challenge. Many pathways represent expected responses to the actions of insulin, including alterations in glycolysis, lipolysis, ketogenesis, and proteolysis. However, we also observed consistent effects of glucose loading on a number of biological pathways that have not previously been well described. Specifically, the neurotransmitter serotonin (and related metabolites) was suppressed more than twofold in response to glucose challenge. In addition, we observed a consistent decrease in the precursors or derivatives related to the B vitamins after OGTT. Lastly, we identified differences in metabolite responses between individuals with and without insulin resistance, indicating that changes in metabolic phenotypes may be unmasked by OGTT in the prediabetic state.

To our knowledge, ours is the largest study to date to describe the physiological changes in metabolites after OGTT. Several smaller studies have reported changes in metabolites with glucose challenge in humans (8,15,16) and animal models (17). Our group previously examined metabolic profiling in response to glucose challenge in healthy university students, with validation in a middle-aged subset of 50 participants of the FHS (8). This study demonstrated significant changes in 18 metabolites, with some differences in metabolite excursions related to baseline fasting insulin levels. We now extend these findings to a far greater sample size and demonstrate significant changes in >90 metabolites representing a number of distinct biological pathways that are involved in the intrinsic biological response to a glucose challenge.

There is a growing body of evidence demonstrating a postprandial or postglucose increase in circulating conjugated bile acids (8,16). Glucose ingestion is known to stimulate the release of cholecystokinin, signaling gallbladder contraction and release of bile into the enterohepatic circulation (18). Experimental evidence suggests that both glucose and insulin rapidly induce cytochrome P-450 enzyme cholesterol 7 α -hydroxylase (*CYP7A1*) gene expression,

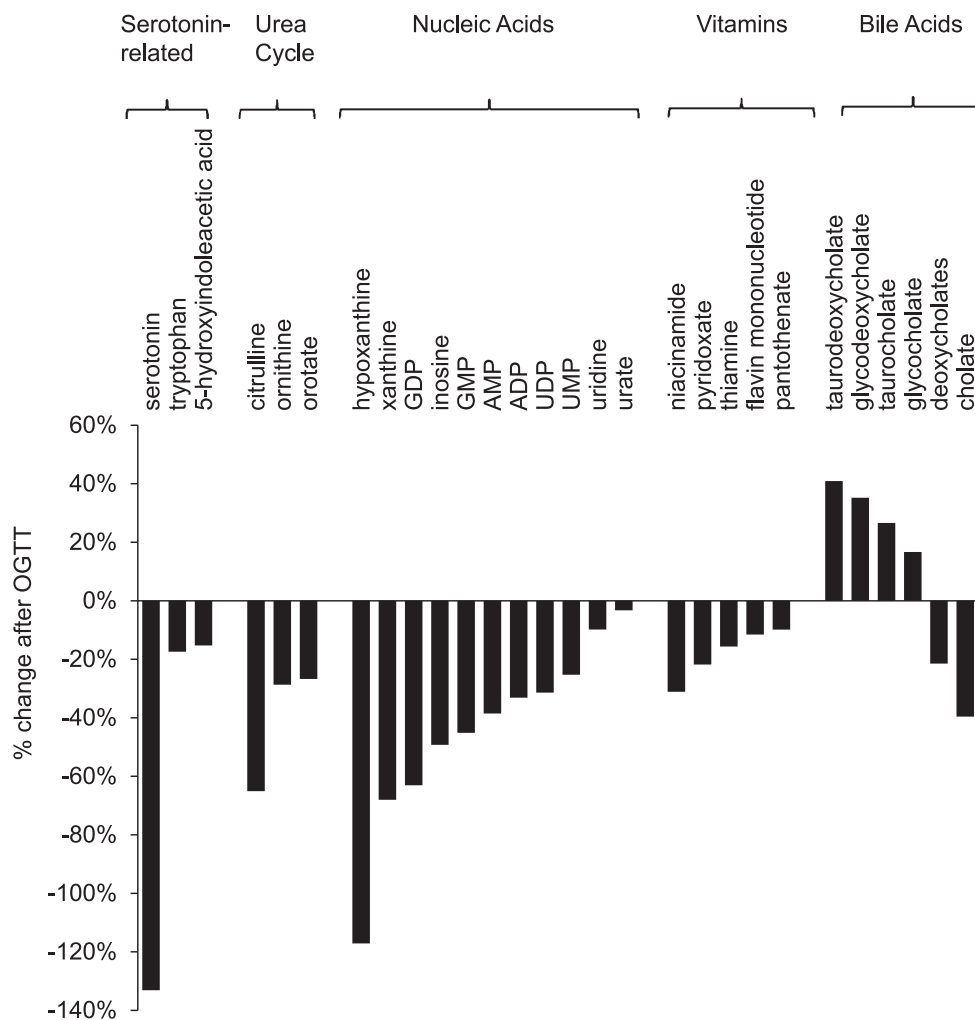


FIG. 3. Metabolite excursions after glucose challenge in pathways that are not previously established in relation to glucose or insulin metabolism. Notable perturbations include inhibition of the serotonin system, decrease in metabolites of the urea cycle, suppression of nucleic acids, decrease in all vitamins of the B superfamily, increase in all conjugated bile acids, and decrease in deconjugated bile acids. % change = $100 \times (\text{up-fold change} - 1)$ or $100 \times (1 - \text{down-fold change})$.

thus augmenting the rate-limiting enzyme of “classical” bile acid synthesis (19,20). Metabolic status also seems to regulate expression of the farnesoid X receptor, a transcription factor that controls bile acid synthesis (21), and expansion of the bile acid pool in turn may be linked to improved glucose homeostasis (22). We not only demonstrate a robust increase in conjugated bile acids in response to glucose challenge but also show a simultaneous decrease in unconjugated bile acids, suggesting that conjugation, a process catalyzed by bile acid CoA:amino acid *N*-acyltransferase (21), may be affected by postprandial increases in glucose and/or insulin. Gut microbiota are known to modify bile acids in a variety of different ways, including deconjugation, dehydroxylation, oxydation, and sulfation (23), and altered bile acid profiles have previously been linked to diabetes in a case-control study (24). It is possible that alterations in gut microbial metabolism may have affected the bile acid profile post-glucose challenge.

One of the notable and unexpected findings of our study was a consistent decrease in vitamins B1, B2, B3, B5, and B6 after glucose challenge. The B vitamins are a group of water-soluble vitamins essential to cellular metabolism. Interestingly, this observation can be considered in the

context of a well-known clinical observation pertaining to thiamine (B1), an essential cofactor for pyruvate dehydrogenase, an enzyme linking glycolysis and the citric acid cycle, as well as α -ketoglutarate dehydrogenase, an important enzyme in the citric acid cycle (25). Thiamine deficiency is the cause of Wernicke’s encephalopathy, an acute neurological disorder seen in the setting of chronic excessive alcohol abuse. It has long been recognized that symptoms of Wernicke’s encephalopathy can be precipitated rapidly after the institution of intravenous glucose therapy in susceptible individuals (26). We now show that a measurable decrease in thiamine occurs even in ostensibly healthy individuals after glucose ingestion. The mechanism for this decrease is unclear, although it may be that increased glycolysis after glucose ingestion drives down circulating plasma thiamine levels. Dynamic changes in the other B vitamins after glucose challenge are of equal interest, as many play essential roles in metabolic pathways affected by insulin action, including amino acid metabolism (B6), gluconeogenesis (B6), the TCA cycle (B5), and various redox reactions (B2 and B3).

We observed a more than twofold decrease in serotonin levels after glucose challenge, as well as a concomitant decrease in its precursor tryptophan and its main metabolite

TABLE 4
Differences in metabolite changes following OGTT between insulin-resistant and non-insulin-resistant groups

	Adjusted for age and sex			Adjusted for age, sex, and BMI		
	Estimate*	SE	P value	Estimate*	SE	P value
β-Hydroxybutyrate	0.280	0.072	<0.001	0.258	0.077	0.001
Isoleucine	0.051	0.014	0.001	0.034	0.015	0.026
Lactate	-0.064	0.022	0.005	-0.040	0.024	0.094
Orotate	-0.079	0.029	0.007	-0.086	0.031	0.006
Pyridoxate	0.043	0.016	0.009	0.035	0.017	0.047

*Estimates represent the difference in metabolite excursions (log-post-OGTT value minus log-pre-OGTT value) after OGTT in insulin-resistant vs. non-insulin-resistant individuals.

5-HIAA. Serotonin is a neurotransmitter that plays an important role in energy and glucose homeostasis, an effect that is mediated via central autonomic pathways in the midbrain, as well as by peripheral release of serotonin from gastrointestinal enterochromaffin cells (27). In experimental studies, genetic deletion of the serotonin receptor

5-HT_{2c} produced insulin resistance and type 2 diabetes (28), and 5-HT_{2c} agonists improved peripheral insulin action (29). In turn, the effect of glucose load on circulating serotonin levels has not been well described. A previous study in 100 healthy individuals measured plasma neurotransmitters in response to OGTT and noted noradrenergic over parasympathetic predominance at 2 h (30). Another study showed postprandial increases in serotonin levels after ingestion of a meal in healthy volunteers (31). These results may contrast with our findings due to differences in the ingested tryptophan content. Serotonin synthesis is sensitive to the availability of tryptophan (32), and it may be that decreases in serotonin follow decreased tryptophan concentrations in response to glucose challenge in our study.

We observed five metabolites with differential changes in response to glucose challenge in insulin-resistant individuals. Interestingly, insulin resistance was associated with a blunted excursion post-OGTT for β-hydroxybutyrate, isoleucine, lactate, and pyridoxate. This confirms that suppression of ketogenesis (β-hydroxybutyrate), proteolysis (isoleucine), and increased glycolysis (lactate) is likely mediated via insulin actions, and that these effects are attenuated in the insulin-resistant state. Furthermore, greater increases in insulin during glucose challenge were associated with larger increases in lactate in individuals with normal HOMA-IR.

Interestingly, there appear to be sex differences in circulating fasting metabolite concentrations and their association with insulin action (33). We now show that there are also sex differences in the dynamic response to glucose challenge, emphasizing the potential role of hormonal factors in the regulation of glucose homeostasis and diabetes pathogenesis (34).

It is thought that impaired fasting glucose predominantly reflects a defect in early insulin secretion during OGTT and hepatic insulin resistance, and impaired glucose tolerance reflects both early and late defects in insulin secretion during OGTT, and mostly insulin resistance at the level of skeletal muscle (35). In exploratory analyses, we found a blunted decrease in β-hydroxybutyrate, α-hydroxybutyrate, tryptophan, and ornithine in individuals with higher 2-h post-OGTT glucose, suggesting that suppression of ketogenesis, proteolysis, and the urea cycle is blunted in these individuals. This may possibly reflect a late-phase defect in insulin secretion, although further studies are needed to substantiate these findings.

Several limitations of the study deserve mention. Although many metabolite changes were in concert with known physiological actions of insulin, it is not possible in our observational study to discern whether perturbations could also have been due to effects of glucose excursion during OGTT. Furthermore, enteric microbiota can influence

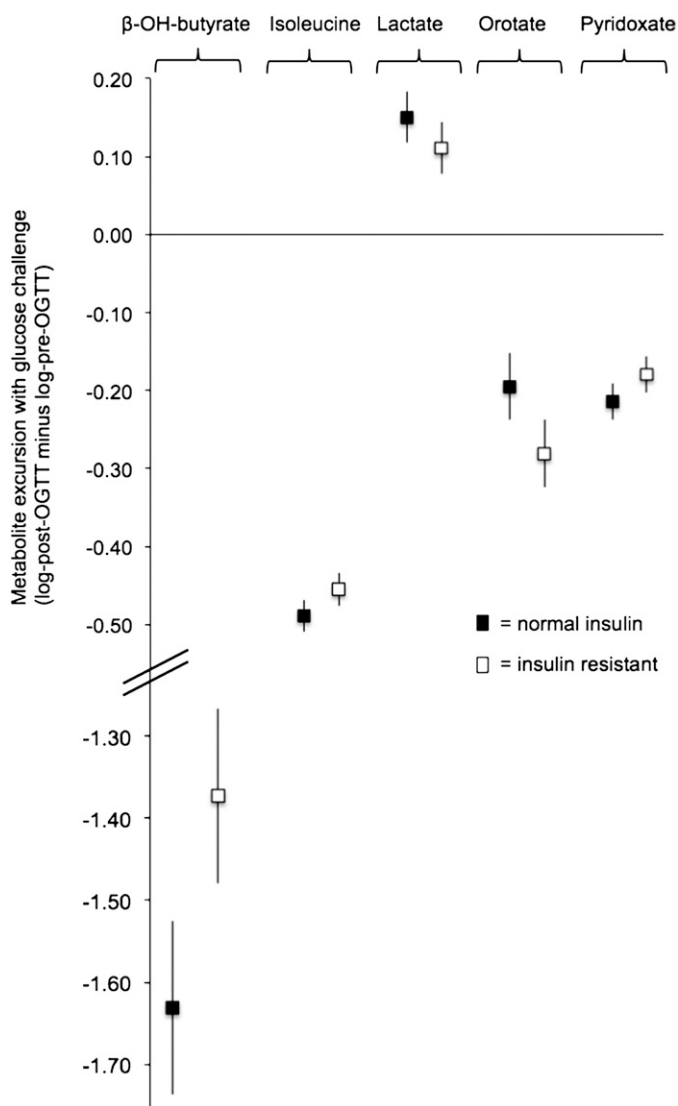


FIG. 4. Metabolite excursions after glucose challenge in participants with and without insulin resistance. Analyses are adjusted for age, sex, and BMI. Error bars represent 95% CIs. Changes for ketogenesis (β-hydroxybutyrate), proteolysis (isoleucine), and glycolysis (lactate) are all blunted in insulin-resistant individuals.

circulating metabolites (36), and it may be that dynamic changes seen with glucose challenge could in part be due to effects on intestinal microbes, rather than pure insulin effects. For example, plasma hippurate concentrations may be elevated due to gut microbial metabolism of phenylalanine (37). It is possible that glucose ingestion stimulated intestinal microbial metabolism, resulting in increased hippurate levels in our study, although other metabolites of microbial metabolism (including propionate, dimethylglycine, trimethylamine, and indoxylsulfate) (8) decreased with glucose challenge. The effect of glucose challenge on gut microbial contributions to changes in plasma metabolites requires further study. Decreases in plasma metabolites could be in part related to a dilutional effect with the volume ingested during glucose challenge. However, in a previous study that diluted the 75-g OGTT by up to threefold, most differences in measured glucose were observed early in the test, with no difference in 2-h values, indicating that this effect may be minimal in our study, which only measured metabolites at the 2-h time point (38). Furthermore, the varying directionality and broad range in change among different analytes would argue against a simple dilutional effect. We used a targeted approach with a combination of LC and tandem MS to identify metabolites. Although this system is less biased than other techniques, such as nuclear magnetic resonance spectroscopy, it allowed for highly specific identification of metabolites. We were not able to provide absolute concentrations for all metabolites, and studies of absolute quantitation of metabolites remain important areas of future investigation. Lastly, the FHS is a predominantly Caucasian middle-aged cohort, and the sample in this study included individuals with a high propensity for the development of diabetes, with a large proportion meeting the criteria for insulin resistance; therefore, our findings may not be generalizable to other populations.

In summary, we found changes in distinct metabolic and biological pathways in response to glucose challenge. Although alterations in glycolysis, lipolysis, ketogenesis, and proteolysis are known effects of insulin action, we also found consistent changes in the serotonergic system and multiple members of the B vitamins after glucose challenge. These findings may further elucidate the metabolic pathways regulating glucose homeostasis. We also identified differences in metabolite responses between individuals with normal insulin and insulin resistance, indicating that changes in metabolic phenotypes associated with the prediabetic state may be unmasked by OGTT. Further studies investigating the distinct biological pathways identified in our study may lead to a better understanding of the adaptive versus maladaptive processes involved in glucose and insulin metabolism.

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J.E.H., R.E.G., and T.J.W. conceived the study, designed experiments, analyzed data, and wrote the manuscript.

M.G.L., R.S.V., A.G., and S.C. helped in experimental design, analyzed data, and assisted in manuscript generation. E.P.R. and C.B.C. developed the metabolic profiling platform, performed MS experiments, analyzed the data, and assisted in manuscript generation. J.C.F. helped in manuscript generation. J.E.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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