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BMI, RQ, Diabetes, and Sex Affect the Relationships Between Amino Acids and Clamp Measures of Insulin Action in Humans

Previous studies have used indirect measures of insulin sensitivity to link circulating amino acids with insulin resistance and identify potential biomarkers of diabetes risk. Using direct measures (i.e., hyperinsulinemic-euglycemic clamps), we examined the relationships between the metabolomic amino acid profile and insulin action (i.e., glucose disposal rate [GDR]). Relationships between GDR and serum amino acids were determined among insulin-sensitive, insulin-resistant, and type 2 diabetic (T2DM) individuals. In all subjects, glycine (Gly) had the strongest correlation with GDR (positive association), followed by leucine/isoleucine (Leu/Ile) (negative association). These relationships were dramatically influenced by BMI, the resting respiratory quotient (RQ), T2DM, and sex. Gly had a strong positive correlation with GDR regardless of BMI, RQ, or sex but became nonsignificant in T2DM. In contrast, Leu/Ile was negatively associated with GDR in nonobese and T2DM subjects. Increased resting fat metabolism (i.e., low RQ) and obesity were observed to independently promote and negate the association between Leu/Ile and insulin resistance, respectively. Additionally, the relationship between Leu/Ile and GDR was magnified in T2DM males. Future studies are needed to determine whether Gly has a mechanistic role in glucose homeostasis and whether dietary Gly enrichment may be an effective intervention in diseases characterized by insulin resistance.

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Prevalence rates for type 2 diabetes (T2DM), prediabetes, metabolic syndrome, and cardiovascular disease have been increasing globally (1) and are responsible for an increased burden of patient suffering and social costs. Insulin resistance is integral in the pathogenesis of these disorders and involves defects in glucose production by the liver and insulin-stimulated glucose uptake and utilization by peripheral tissues. While obesity is associated with insulin resistance, general adiposity explains only a minor portion of variability in insulin resistance among nondiabetic individuals (2,3).

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Numerous studies have found that insulin resistance and diabetes are associated with fat accumulation in the visceral compartment, skeletal muscle, and liver tissue (4–7). Recent research suggests that amino acids may also be important in the development of insulin resistance as alterations in circulating levels of several amino acids, including branched-chain amino acids (BCAA) and aromatic amino acids (AAA), are associated with obesity (8–11) and insulin resistance (8,10) and identified as the best early predictor for the future development of diabetes (12). Moreover, baseline levels of BCAA, AAA, and related metabolites are prognostic for improvement in insulin sensitivity in response to a dietary/behavioral intervention (13) and are tightly correlated with improvement in glucose homeostasis and insulin sensitivity after bariatric surgery (14). While these studies have focused primarily on BCAA and AAA, other amino acids may also be relevant in the development of insulin resistance and T2DM (11,15). Furthermore, multiple intrinsic factors (e.g., amino acid metabolism, protein metabolism [16]), hormonal changes, and extrinsic factors (e.g., dietary intake, physical activity [11]) can contribute to changes in amino acid concentrations (17).

Reported relationships between amino acid concentrations and insulin resistance in humans have primarily involved surrogate measures of insulin sensitivity (e.g., homeostasis model assessment of insulin resistance [HOMA-IR]) (10,18,19), which may limit the accuracy of their predictive value (20). The objective of the current study was to examine, for the first time, the relationships between amino acid levels and the gold standard measure of insulin sensitivity, the hyperinsulinemic-euglycemic clamp, in human subjects. This technique quantifies whole-body insulin action under conditions where the bulk of the insulin-stimulated glucose uptake is into skeletal muscle (21), which is responsible for the vast proportion of in vivo glucose uptake in response to insulin. We have confirmed a relationship between BCAA and insulin resistance and, importantly, have demonstrated a major signal for glycine (Gly), as well as the influence of BMI, race, and respiratory quotient (RQ) on these relationships.

RESEARCH DESIGN AND METHODS

Subjects were recruited from advertisements and word-of-mouth referrals and sequentially enrolled. An effort was made to have equal enrollment of European and African Americans such that only African Americans were entered into the study after the full complement of European Americans had been recruited. The final study group comprised 124 volunteers (63 European American and 60 African American) with ages between 21 and 59 years.

None of the volunteers had cardiovascular, renal, or hepatic disease, and all were chemically euthyroid. No subjects were pregnant or taking pharmacological agents known to affect carbohydrate or lipid metabolism. Weight was stable (±3%) for ≥3 months before study, BMI was between 21 and 46 kg/m^2, and none of the study subjects engaged in regular exercise. Race was determined by self-report. Premenopausal females were studied between days 3 and 10 of the menstrual cycle. Studies were performed in the morning after a 12-h fast. Subjects were equilibrated on an isocaloric diet with macronutrient composition of 30% fat, 55% carbohydrate, and 15% protein for 3 days prior to studies.

Protocols were approved by the University of Alabama at Birmingham Institutional Review Board. Written informed consent was obtained from every subject.

Insulin Action

In vivo insulin action was assessed as maximal insulin responsiveness via hyperinsulinemic-euglycemic glucose clamp technique at a maximally effective steady-state serum insulin concentration as previously described (6,22,23). Briefly, glucose and KPO4 were administered through a catheter inserted into the brachial vein. A dorsal hand vein was cannulated in a retrograde manner and kept in a warming device (65°C) to provide arterialized venous blood for sampling. Regular insulin (Humulin; Eli Lilly, Indianapolis, IN) was administered at 200 mU $\cdot$ m^{-2} $\cdot$ min^{-1} to produce a mean steady-state insulin concentration of 501 ± 20 μIU/mL. This level is maximally effective for suppressing hepatic glucose production and has been shown to predominantly reflect maximally stimulated skeletal muscle glucose uptake under these experimental conditions (21). Serum glucose was clamped at 90 mg/dL for a minimum of 3 h within a <5% coefficient of variation. Maximal glucose uptake was determined as the mean glucose infusion rate over the final three 20-min intervals. Whole-body glucose uptake was calculated as the glucose infusion rate corrected for changes in the glucose pool size, assuming a distribution volume of 19% body weight and a pool fraction of 0.65. Glucose uptake was normalized per kilogram lean body mass to yield the glucose disposal rate (GDR). Lower GDR values indicate insulin resistance. HOMA-IR was calculated from fasting plasma insulin and glucose levels with the following formula: HOMA-IR = plasma insulin (μU/mL) × plasma glucose (mmol/L)/22.5 (24). Higher HOMA-IR values indicate insulin resistance.

Amino Acids Measured by Mass Spectrometry

Fasting serum samples, collected prior to initiating the clamp procedures, were analyzed for amino acid concentrations by flow-injection tandem mass spectrometry as described (25). Sixteen amino acids were measured using stable isotope dilution techniques: alanine (Ala), glycine (Gly), valine (Val), leucine/isoleucine (Leu/Ile), phenylalanine (Phe), tyrosine (Tyr), glutamate/glutamine (Glx), aspartate/asparagines (Asx), arginine (Arg), citrulline (Cit), histidine (His), methionine (Met), ornithine (Orn), proline (Pro), and serine (Ser). Sample preparation methods were performed as previously described (10,25). Briefly, samples were equilibrated with a cocktail of

internal standards and deproteinated by precipitation with methanol, and then aliquoted supernatants were dried and then esterified with hot, acidic n-butanol. The data were acquired using a Micromass Quattro micro-TM system equipped with a model 2777 autosampler, a model 1525 μ HPLC solvent delivery system, and a data system controlled by MassLynx 4.0 operating system (Waters, Milford, MA).

Anthropometric and Body Composition Measurements

BMI was calculated as weight in kilograms divided by the square of height in meters. Waist and hip circumferences were measured using a tension-controlled tape measure. Dual-energy X-ray absorptiometry (DEXA), using Prodigy (GE Medical Systems LUNAR, Madison, WI) with software version 6.10.029 (enCORE 2002), provided total body fat and lean body mass independent of bone mass (26).

Statistical Analyses

Differences in variables of interest were compared using univariate ANOVA and reported as mean ± SD. Principle components analysis was performed to identify mechanistic-related groupings among the 16 amino acids. Partial correlations controlled for age, sex, race, and BMI were used to examine the relationships among amino acids (including identified components) and insulin action in the overall population and also stratified by diabetes status and BMI. Sensitivity analyses were performed to detect race or sex influence in the correlations. In analyses stratified by race or sex, these stratification variables were not used as controlling variables.

Stepwise multiple regression analyses were used to determine which, if any, amino acids were most predictive of GDR in the overall cohort, as well as in both BMI groups and T2DM patients. The most predictive amino acids revealed in the regression analysis were then used in additional stepwise multiple regression analyses to assess the predictability of GDR, along with RQ, BMI, sex, and race. Missing data were handled by pairwise deletion. Analyses were performed using SPSS 20.0 for Windows (SPSS, Chicago, IL), and differences were accepted as significant at $P < 0.05$.

RESULTS

Descriptive characteristics of study subjects, stratified by diabetes status and insulin sensitivity, are delineated in Table 1. As expected, T2DM subjects were most insulin resistant (IR); however, nondiabetic subjects displayed a wide variability in insulin responsiveness and were categorized into insulin-sensitive (IS) and -resistant subgroups based on values above and below the median value of GDR. Differences in insulin responsiveness between T2DM and IR, compared with IS, were further observed from differences in HOMA-IR. T2DM (vs. IS and IR) also displayed elevated fasting glucose and reduced HDL compared with the other two groups, while the IS group displayed the lowest fasting glucose and

Table 1—Descriptive characteristics of study subjects

		Non-T2DM		
	All subjects	IS	IR	T2DM
N	124	61	32	31
Race	51% EA, 48% AA	46% EA, 53% AA	47% EA, 53% AA	64% EA, 36% AA
Sex (% male)	41	34	47	48
Age (years)	42 ± 10	41 ± 9	40 ± 11	45 ± 9
GDR (mg/kg LBM/min)	12.4 ± 4.7	16.1 ± 3.1	9.6 ± 2.0†	7.4 ± 2.4†‡
HOMA-IR	4.72 ± 3.71	3.18 ± 1.57	5.51 ± 2.95†	6.97 ± 5.68†
Waist (cm)	99 ± 14	94 ± 12	105 ± 14†	103 ± 14†
BMI (kg/m^2)	31.0 ± 5.0	30.2 ± 5.0	33.3 ± 6.0§	30.4 ± 6.0
Fat (%)	37.6 ± 10.0	37.5 ± 11.0	42.3 ± 8.0§	32.8 ± 8.0‡
Lean body mass (kg)	52.3 ± 12.0	49.0 ± 10.0	54.0 ± 12.0	57.1 ± 14.0§
REE (kcal/day)	1608 ± 298	1542 ± 255	1661 ± 314	1684 ± 335
Resting RQ	0.85 ± 0.06	0.86 ± 0.06	0.84 ± 0.07	0.82 ± 0.04†
FFA (mmol)	0.53 ± 0.24	0.51 ± 0.22	0.53 ± 0.16	0.66 ± 0.47
HDL (mg/dL)	46.0 ± 18.7	53 ± 21	43 ± 14§	35 ± 10†‖
LDL (mg/dL)	119.4 ± 38.2	117 ± 41	122 ± 37	120 ± 36
Fasting insulin (μIU/ml)	17 ± 11	14 ± 7	23 ± 11†	15 ± 15
Fasting glucose (mg/dL)	122 ± 65	90 ± 9	97 ± 11†	214 ± 77†‡

Data are means ± SD unless otherwise indicated. AA, African American; EA, European American; FFA, free fatty acids; REE, resting energy expenditure. §$P < 0.05$ compared with IS. †$P < 0.01$ compared with IS. ‡$P < 0.01$ compared with IS. ‖$P < 0.05$ compared with IS.

highest HDL. The mean BMI was similar among IS, IR, and T2DM subgroups. Waist circumference was lowest in the IS group.

Compared with IS, IR had reduced levels of Gly, Ser, and Cit but elevated Glx (Table 2). T2DM had reduced Gly and His, but elevated Leu/Ile, Val, Asx, and Glx compared with IS, and elevated Leu/Ile and Asx and reduced His levels compared with IR.

Principle components analysis of the 16 amino acids yielded two extracted components. Component 1 included the BCAAs, Leu/Ile and Val (46.4% variance), and component 2 included Gly and Ser (41.2% variance). Together, these two components explained 87.6% of the variance in the data set.

BMI correlated with GDR in the overall population ($r = -0.18$, $P < 0.05$). Therefore, statistical analyses were controlled for BMI where appropriate. For the entire cohort (i.e., IS, IR, and T2DM combined), Gly had the strongest (positive) correlation with GDR when age, BMI, sex, and race and were controlled for, while Leu/Ile had the strongest negative correlation (Fig. 1*A* and *B*). These relationships with GDR were closely followed by components 2 (positive relationship) and 1 (negative relationship [Fig. 1*A*]). Sensitivity analyses were performed to explore whether sex, race, or diabetes impacted the correlations between GDR and individual amino acids (Gly, Ser, Leu/Ile, and Val) and the two principle components (Table 3): while only slight racial differences were detected, stronger sex differences were revealed. The relationships between GDR, the amino acids, and their respective components were strong and significant in the female population but attenuated in the male population, with the exception of Gly, which remained strong in both sexes. The relationship between Leu/Ile and GDR was intensified in the T2DM males ($r = -0.726$, $P = 0.017$) when the data were stratified by sex. It is important to note, however, that the sample size in this group is only 13, so this result may not be generalizable to other populations. The positive relationship between GDR and Gly was strong in normoglycemic (i.e., IS and IR) subjects but attenuated in T2DM, while the opposite phenomenon occurred with Leu/Ile: the relationship was strong in T2DM but attenuated in normoglycemic subjects. Due to these differences, subsequent analyses consider T2DM separately, and all analyses are controlled for sex and race.

For examination of the influence of obesity on the relationships between GDR and amino acids, subjects were stratified by BMI and diabetes status and partial correlation analyses controlled for age, sex, and race were performed (Fig. 2). Descriptive characteristics and serum amino acid contents for these groups are provided in Supplementary Tables 1 and 2, respectively. In nonobese subjects (BMI <30 kg/m^2), Gly and component 2 were positively correlated to GDR, while Leu/Ile and component 1 were negatively related. In obese individuals (BMI ≥ 30 kg/m^2), Gly, component 1, and six other amino acids were positively related to GDR, while Leu/Ile and component 2 were not related. Thus, BMI was an important determinant as to whether BCAAs were associated with insulin resistance, while Gly and component 2 remained correlated with insulin action across the BMI spectrum. Finally, in T2DM subjects, only Leu/Ile was significantly correlated with GDR (Fig. 2).

For determination of whether relationships between amino acid levels and GDR were affected by differences

Table 2—Circulating amino acid levels in IS, IR, and T2DM subgroups

	Non-T2DM		
	IS	IR	T2DM
N	61	32	31
Amino acids (μmol/L)			
Gly	306.8 ± 93.9	257.0 ± 58.3†	246.8 ± 61.8†
Leu/Ile	163.5 ± 36.9	180.8 ± 37.0	204.4 ± 36.0†‡
Ala	350.4 ± 106.3	350.5 ± 130.3	357.1 ± 118.0
Ser	111.8 ± 26.6	98.7 ± 19.7§	107.3 ± 20.4
Pro	195.1 ± 65.9	189.2 ± 58.3	185.8 ± 57.6
Val	254.7 ± 55.5	271.9 ± 40.9	286.8 ± 52.2§
Met	18.6 ± 4.6	18.2 ± 3.5	16.7 ± 3.8
His	67.4 ± 13.5	66.5 ± 12.1	59.2 ± 10.2†‡
Phe	73.0 ± 14.8	75.2 ± 13.2	75.6 ± 12.4
Tyr	74.5 ± 22.0	83.0 ± 25.3	77.9 ± 28.1
Asx	82.9 ± 45.6	85.7 ± 42.7	121.2 ± 62.1†‡
Glx	83.8 ± 21.7	96.5 ± 21.3§	103.5 ± 22.0†
Orn	49.9 ± 13.6	50.6 ± 15.6	53.7 ± 12.7
Cit	33.5 ± 9.4	29.0 ± 7.7§	31.7 ± 8.8
Arg	83.9 ± 24.0	75.9 ± 20.7	79.7 ± 21.9

Data are means ± SD. §$P < 0.05$ compared with IS. †$P < 0.01$ compared with IS. ‡$P < 0.05$ compared with IS.

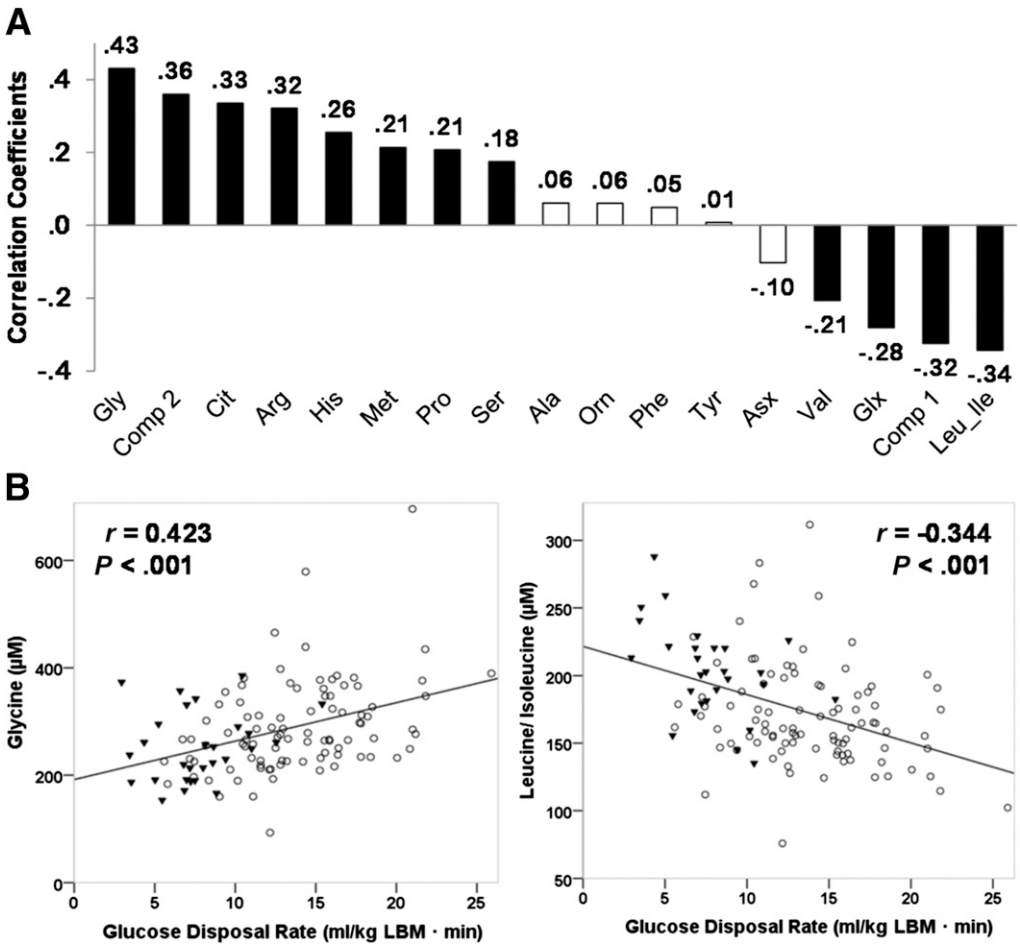

Figure 1—*A*: Relationships between insulin sensitivity, measured by hyperinsulinemic-euglycemic clamp, and circulating amino acid concentrations (N = 120). Correlations are controlled for age, BMI, sex, and race. ■, significant correlation; □, nonsignificant correlations. *B*: Scatter plot showing the correlation between insulin sensitivity and amino acids Gly (*left*) and Leu/Ile (*right*). ○, normoglycemic subjects; ▼, those with T2DM. During the clamp studies, plasma glucose was clamped at 90 mg/dL in all subjects within a coefficient of variation <5%. The mean steady-state serum insulin level achieved during the clamps at the indicated infusion rate was 501 ± 20 μU/mL. LBM, lean body mass.

in fuel preference (i.e., fat vs. carbohydrate oxidation), correlation analyses were performed after stratification into subgroups with low and high resting RQ values (below and above the median RQ value) and with controlling for age, race, sex, and BMI (Fig. 3). A strong, negative relationship between GDR and Leu/Ile was observed in subjects with an RQ below the median RQ, while no relationship was detected in those with an RQ higher than the median RQ. In contrast, positive and statistically significant associations were observed between GDR and Gly in both RQ groups.

Our studies have indicated that BMI, resting RQ, and sex can affect the correlations between amino acid levels and insulin responsiveness. Therefore, stepwise multiple regression analyses were performed to determine the extent to which these factors acted independently to determine insulin action measured by GDR (Table 4). In the overall cohort and in the BMI <30 kg/m^2 subgroup, only Leu/Ile and Gly entered the regression equation with statistical significance and exerted independent effects that predicted the GDR. In the BMI ≥ 30 kg/m^2 group, Gly, but not Leu/Ile, in combination with RQ, sex, and BMI, were independently predictive of GDR. In the T2DM group, only Leu/Ile was predictive of GDR (Table 4).

The correlations between HOMA-IR and both Gly (r = -0.211, P = 0.021) and component 2 (r = -0.204, P = 0.026) were weaker than the observed correlations with GDR (above). However, the relationships between HOMA-IR and Leu/Ile (r = 0.341, $P < 0.0001$) and component 1 (r = 0.366, $P < 0.0001$) were similar to the results from the hyperinsulinemic clamp (reported above).

DISCUSSION

This is the first study to examine the relationship between circulating amino acids and insulin resistance in humans using the gold standard measure of whole-body

Table 3—Impact of diabetes status, sex, and race on the correlations between GDR and amino acids GLY, Leu/Ile, Ser, and Val

		Gly		Leu/Ile		Ser		Val		Component 1‖		Component 2¶	
	n	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
All subjects†	120	0.422	0.000	−0.344	0.000	0.173	0.064	−0.207	0.026	−0.324	0.000	0.358	0.000
Normoglycemic†	93	0.418	0.000	−0.120	0.262	0.296	0.005	−0.045	0.678	−0.133	0.213	0.403	0.000
T2DM†	27	0.106	0.629	−0.469	0.024	−0.030	0.893	−0.259	0.252	−0.362	0.090	0.084	0.702
All women‡	71	0.484	0.000	−0.454	0.000	0.360	0.003	−0.330	0.006	−0.454	0.000	0.489	0.000
All men‡	49	0.308	0.037	−0.154	0.308	−0.147	0.330	−0.020	0.893	−0.107	0.478	0.110	0.467
Non-DB women‡	57	0.468	0.000	−0.251	0.068	0.354	0.009	−0.143	0.303	−0.267	0.051	0.457	0.001
Non-DB men‡	36	0.295	0.095	0.079	0.661	0.135	0.452	0.119	0.511	0.082	0.652	0.250	0.160
T2DM women‡	14	0.534	0.091	−0.280	0.405	0.474	0.141	0.049	0.886	−0.143	0.675	0.654	0.029
T2DM men‡	13	−0.235	0.513	−0.726	0.017	−0.237	0.509	−0.678	0.031	−0.688	0.028	−0.182	0.615
All EA§	59	0.409	0.002	−0.430	0.001	0.042	0.759	−0.290	0.029	−0.392	0.003	0.299	0.024
All AA§	59	0.482	0.000	−0.231	0.086	0.338	0.011	−0.115	0.400	−0.241	0.073	0.463	0.000

AA, African American; EA, European American; Non-DB, normoglycemic. †Controlled for age, BMI, race, and sex. ‡Controlled for age, BMI, and race. §Controlled for age, BMI, and sex. ‖Component 1 includes Leu/Ile and Val. ¶Component 2 includes Gly and Ser.

insulin action, the hyperinsulinemic-euglycemic clamp technique. At maximally effective steady-state serum insulin concentrations, the clamp technique quantifies whole-body insulin action on glucose uptake with the bulk of insulin-stimulated glucose uptake occurring into skeletal muscle (21). Therefore, the current study is the first to allow an assessment of circulating amino acid concentrations in relation to insulin action largely in skeletal muscle, the critical tissue for insulin action defects that mediate the clinical manifestations of insulin resistance. To test this hypothesis, previous studies have used obesity (i.e., BMI) or surrogate indices of insulin sensitivity involving mathematical derivations of fasting glucose and insulin; however, neither BMI nor these indices of insulin sensitivity display robust correlations with clamp measures of insulin responsiveness (20). For example, measures of general adiposity, such as BMI, only explain ~8% of

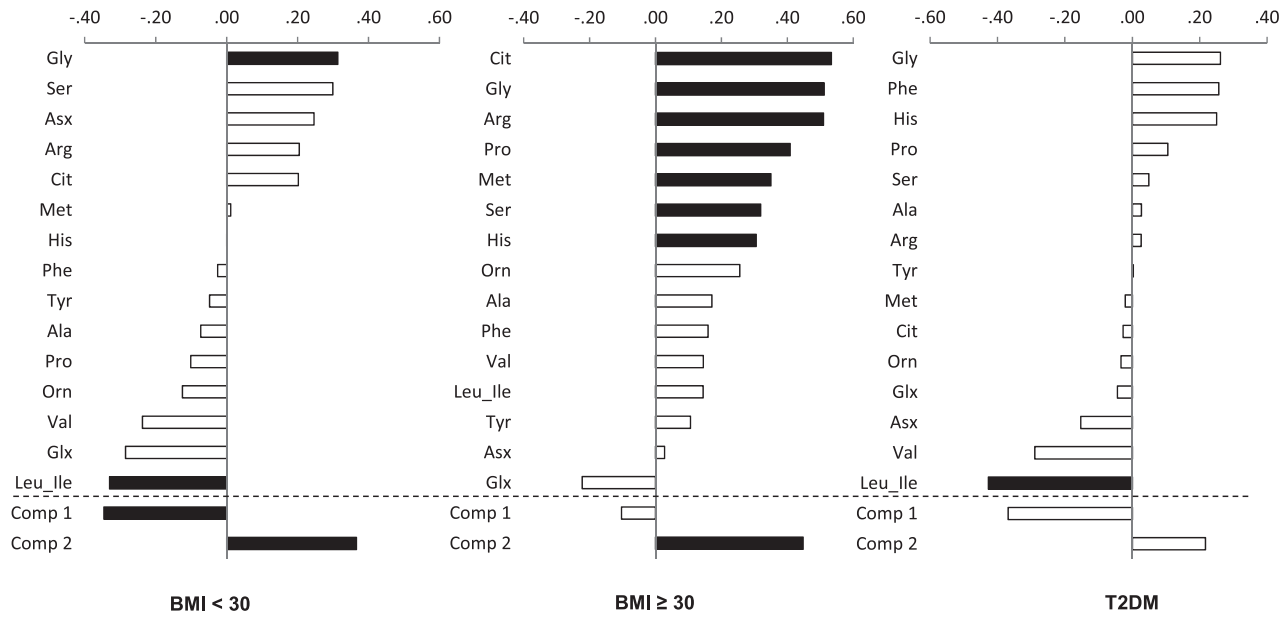

Figure 2—Impact of BMI and T2DM on the relationships between amino acid levels and insulin sensitivity assessed by clamp. *x*-Axis values represent correlation coefficients. ■, significant correlation; □, nonsignificant correlations. Component (Comp) 1, Leu/Ile and Val; Comp 2, Gly and Ser.

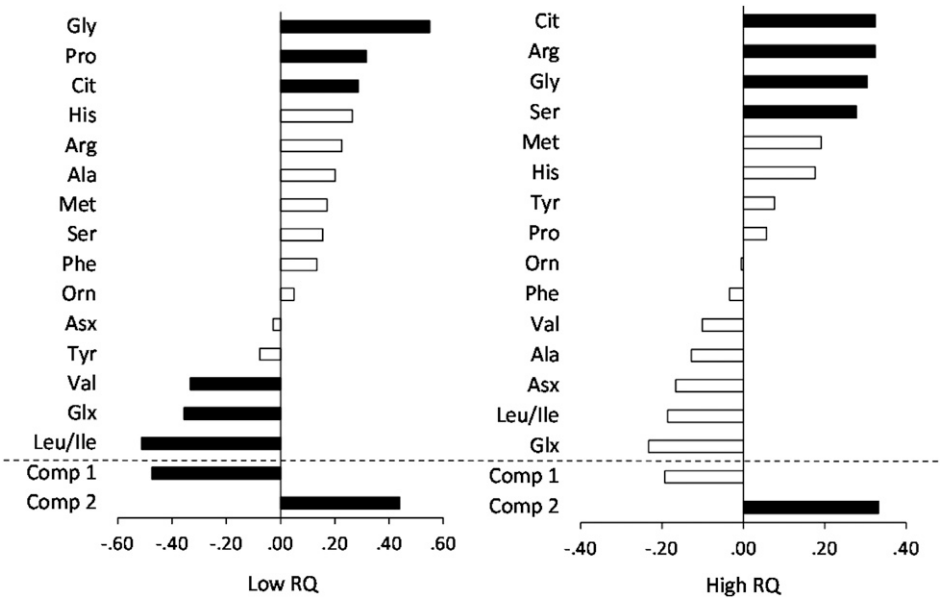

Figure 3—Impact of resting RQ on the relationships between amino acid levels and insulin sensitivity assessed by clamp. *x*-Axis values represent correlation coefficients. ■, significant correlation; □, nonsignificant correlations. Component (Comp) 1, Leu/Ile and Val; Comp 2, Gly and Ser.

individual differences in insulin sensitivity when assessed by hyperinsulinemic-euglycemic clamp, while measures of central fat distribution, such as trunk–to–leg fat ratio, can explain 20% of the variance (27). Because of the current study design, we have been able to advance our understanding of amino acid metabolomic profiles and insulin resistance in humans. Specifically, while we have confirmed the previous association between insulin resistance and elevated BCAAs, we have now shown that these relationships can be dramatically affected by BMI, RQ, and T2DM. Importantly, a new observation is the uniquely strong and persistent correlation between Gly and insulin action among nondiabetic individuals regardless of BMI and RQ.

In our cohort, multiple amino acids had strong associations with GDR over a broad range of insulin sensitivity. In particular, Gly emerged as the amino acid with the strongest positive correlation with insulin action and Leu/Ile with the strongest negative correlation. Furthermore, principle components analysis empirically identified only two components significantly correlated with GDR; component 1 with Gly and Ser was positively associated with GDR and component 2 with the BCAAs, which was negatively associated. However, the strength of these associations was again significantly influenced by BMI, RQ, diagnosed T2DM, and sex.

Gly was found to have a strong positive correlation with GDR in both lean and obese subgroups and in subgroups with low and high resting RQ, raising the

Table 4—Stepwise multiple regression analyses assessing the independent effects of amino acids, RQ, and BMI as predictors of insulin sensitivity

Group	Stepwise multiple regression models	R^2	SE of the estimate	R^2 change	*F* change	Significance of *F* change
All subjects (*N* = 120)	1 Leu/Ile	0.177	4.23	0.178	25.4	0.000
	2 Leu/Ile, Gly	0.39	3.68	0.208	39.4	0.000
BMI <30 kg/m^2 (*N* = 43)	1 Leu/Ile	0.094	4.04	0.094	4.271	0.045
	2 Leu/Ile, Gly	0.322	3.54	0.228	13.46	0.001
BMI ≥30 kg/m^2 (*N* = 49)	1 Gly	0.276	3.22	0.276	17.902	0.000
	2 Gly, RQ	0.365	3.05	0.089	6.456	0.014
	3 Gly, RQ, sex	0.423	2.94	0.058	4.542	0.039
	4 Gly, RQ, sex, BMI	0.474	2.83	0.051	4.291	0.044
T2DM (*N* = 27)	1 Leu/Ile	0.234	2.17	0.234	7.626	0.011

question as to whether Gly was a passive marker or exerted a causal effect to enhance insulin action. However, the mechanisms by which Gly interacts with GDR have yet to be elucidated. Data from studies involving rodents and cultured cells are consistent with a causal role. C57BL/6J mice, with diet-induced obesity and depressed glucose infusion rates, have reduced levels of Gly (28). Gly administration has been shown to suppress proinflammatory adipokines (e.g., tumor necrosis factor-α, interleukin [IL]-6) and increase adiponectin in 3T3-L1 adipocytes (29,30) and in lean mice (30,31). Additionally, in obese mice, Gly suppressed TNF-α and IL-6 gene expression in fat tissue and reduced IL-6, resistin, and leptin protein levels (29,30). Gly was further found to improve glucose tolerance in lean, but not obese, mice (29,30). Gly is also a substrate for glutathione biosynthesis, raising the possibility that high Gly could enhance antioxidant defense. While speculative, these findings indicate that Gly could enhance glucose homeostasis and perhaps insulin action by influencing adipose tissue biology and inflammatory cytokine production, although, again, favorable changes in glucose metabolism in vivo were only observed in lean and not obese mice. Further research is warranted to determine the role of Gly on insulin sensitivity and inflammation in metabolic dysfunction.

In humans, the current data are consistent with previous observations, including a clear decrease in Gly levels in obese IR subjects compared with lean control subjects (10), reduced levels in Japanese patients with metabolic syndrome that were then increased after lifestyle modification (11), an increase in Gly levels in response to bariatric surgery (14), and decreased Gly levels in IR offspring of two T2DM parents (15). In addition, exercise training leading to an increase in insulin sensitivity measured by the frequently sampled intravenous glucose tolerance test was associated with increments in Gly and Pro levels (18). While we have demonstrated a quantitative relationship with GDR values, it remains unclear in humans based on the current study and existing literature whether Gly is causally related to insulin action; nevertheless, the data support a trial assessing effects of dietary Gly enrichment in IR patients.

In patients with T2DM, Gly levels were significantly reduced compared with nondiabetic subjects, and the positive correlation was weakened and no longer statistically significant. Since insulin resistance in T2DM is exacerbated by hyperglycemia, with a consequent increase in glucose metabolism via the hexosamine biosynthetic pathway (32), it is tempting to speculate that Gly does not actively participate in, or protect against, glucose-induced insulin resistance. In cultured adipocytes, the presence of amino acids such as Gly, Thr, and L-glutamine are permissive for the full expression of insulin resistance induced by high glucose (33). Even so, in this scenario, the severity of the component of insulin resistance due to hyperglycemia would not be quantitatively related to the plasma Gly level in vivo.

In agreement with previous studies, we were able to confirm a negative relationship between BCAAs, including Leu/Ile and the principal component comprising both Leu/Ile and Val, and insulin action. In the current study, this relationship was established using clamp measures that we assume reflect insulin action in skeletal muscle. Furthermore, this relationship was modulated by BMI, resting RQ, and sex. Leu/Ile had a strong negative correlation with GDR in the nonobese subgroup and in T2DM patients but not in the nondiabetic obese subgroup. Thus, the presence of obesity obviated the relationship between BCAAs and insulin action, even though the obese individuals had higher levels of BCAA compared with the nonobese subgroup. Interestingly, observed relationships between BCAA and GDR were influenced by sex, such that the relationship between Leu/Ile and GDR was strengthened in the males.

Newgard et al. (10) demonstrated in rodents that BCAA supplementation of a high-fat diet contributes to insulin resistance; however, this was not observed when BCAAs were supplemented into normal chow. This suggests that the availability or preference of fat as a fuel source may be a driver of the relationship between BCAA and insulin resistance. To explore this possibility in humans, we performed analyses in nondiabetic subjects stratified by low and high resting RQ values. Leu/Ile correlated with GDR in the low-RQ group, who prefer oxidation of fat to maintain resting energy expenditure, but not in subjects with high-RQ, who prefer carbohydrates as a fuel source. In T2DM patients, mean RQ was lower than in the nondiabetic subgroups, and only Leu/Ile exhibited a significant and negative correlation with GDR. The data indicate that BCAAs are related to insulin action only under conditions of high lipid metabolism whether induced by high-fat feeding in rodents or low RQ in humans. In the current study, no difference in the mean RQ value could be detected in the obese versus nonobese subgroup; therefore, differences in resting fuel preference could not explain the loss of association between Leu/Ile and GDR in the obese subjects. In fact, in multiple regression models, obesity and RQ exerted independent effects to modulate this relationship.

In previous studies, subjects with metabolic syndrome (6,8) and obesity (8) have been reported to have elevated BCAA concentrations. Additionally, infusion of amino acids during a hyperinsulinemic-euglycemic clamp induced insulin resistance in healthy young males (34). Overnutrition involving a high-protein diet is also associated with insulin resistance (35). Together, these studies suggest that BCAAs could be causally related to insulin resistance. Elevated BCAA may result from the following: decreased BCAA metabolism in adipose tissue or skeletal muscle; reduced insulin-stimulated, anti-proteolytic mechanisms within the skeletal muscle; increased dietary intake; decreased physical activity; or

increased autophagy. A potential mechanism for the observed negative relationship between Leu/Ile and action in IR and T2DM subgroups could involve an impaired ability of insulin to inhibit skeletal muscle proteolysis, leading to an increase in BCAA in the skeletal muscle pool (36). It is unclear, however, why higher Leu/Ile levels, in obesity, are related to GDR in nonobese but not in obese humans. One possibility is an adipose tissue cut point after which the impact of BCAAs on insulin action is diminished. In any case, the higher levels of Leu/Ile in the obese appear to exist independent of changes in insulin action.

While the relationship between Gly and GDR remained strong when the data were stratified by sex, the general lack of correlations between GDR and amino acids in the males is potentially due to fewer males in the analyses. Support for this inference comes from the additional analyses after stratification by sex in the BMI and T2DM subgroups; however, the relationship between GDR and Leu/Ile was intensified in the T2DM males. Future research is warranted to determine whether sex influences the relationships between amino acids and GDR and the associated mechanisms.

When HOMA-IR was used as the measure of insulin sensitivity, the relationship with Gly was attenuated, while the relationship with Leu/Ile remained strong. Differences in the magnitude of the identified relationships between amino acids, especially Gly, and markers of insulin action from the clamp versus surrogate HOMA-IR measurement may be due to the fact that the maximally stimulated clamp effectively shuts down hepatic glucose production and largely reflects insulin action in skeletal muscle (21), while HOMA reflects both hepatic and muscle glucose metabolism. The correlation between GDR and HOMA in our data was -0.461 ($P < 0.0001$), which is in agreement with a previous study where we demonstrated that caution is warranted in the interpretation of data using insulin sensitivity indices such as homeostasis model assessment (20).

In summary, metabolomic amino acid profiles and hyperinsulinemic clamps performed in nondiabetic and T2DM individuals over a broad range of GDR and BMI have demonstrated that *1*) the amino acid with the most robust positive correlation with insulin action is Gly and strongest negative correlation is Leu/Ile; *2*) the association between Gly and insulin action remains strong regardless of BMI, RQ, or sex but is weakened and nonsignificant in T2DM; *3*) the relationship between Leu/Ile and insulin resistance is profoundly influenced by BMI, fuel metabolism, and sex: Leu/Ile is associated with insulin resistance in the nonobese and T2DM subjects only and intensified in T2DM males; and *4*) increased resting fat metabolism (i.e., low RQ) and obesity independently promote and negate the association between Leu/Ile and insulin resistance, respectively. While it is unlikely that amino acid levels are the sole contributors to the observed differences in GDR, future research identifying the metabolic disturbances that link Gly and Leu/Ile with GDR is necessary to fully understand the pathogenesis of insulin resistance and diabetes. Additionally, future studies are needed to determine whether Gly has a mechanistic role in glucose homeostasis and whether dietary Gly enrichment may be an effective intervention in diseases characterized by insulin resistance.

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