Central Adiposity-Induced Plasma Free Amino Acid Alterations Are Associated with Increased Insulin Resistance in Healthy Singaporean Adults

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ABBREVIATIONS

AAAs, aromatic amino acids; Ala, alanine; Arg, arginine; Asp, aspartic acid; BCAAs, branched-chain amino acids; BMI, body mass index; CVD, cardiovascular disease; Cys, cysteine; DBP, diastolic blood pressure; DM, diabetes mellitus; EAAs, essential amino acids; FBG, fasting blood glucose; FSI, fasting serum insulin; GAAs, glucogenic amino acids; Gln, glutamine; Glu, glutamate; Gly, glycine; HC, hip circumference; HDL, high-density lipoprotein; His, histidine; HOMA-IR, homeostasis model assessment of insulin resistance; IFG, impaired fasting glucose; Ile, isoleucine; LDL, low-density lipoprotein; Leu, leucine; Lys, lysine; Met, methionine; mTOR, mammalian target of rapamycin; NEAAs, non-essential amino acids; Orn, ornithine; PFAA, plasma free amino acid; Phe, phenylalanine; PKC, protein kinase C; Pro, proline; SAAs, sulfur-containing amino acids; SBP, systolic blood pressure; Ser, serine; T2D, type 2 diabetes; TC, total cholesterol; TCA, trichloroacetic acid; TG, triglycerides; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; WC, waist circumference; WHR, waist-to-hip ratio.

ABSTRACT

Objectives: Recent metabolomics technique reveals a plasma free amino acid (PFAA)-based metabolite signature that is suggestive of altered PFAAs being an early manifestation of obesity-related insulin resistance. However, the PFAA profiles within non-obese, but more insulin resistant Asians are not well researched. Compared to Caucasians, Asian populations have more central adiposity, which is generally regarded as metabolically more adverse, but the underlying mechanisms remain unclear. In the present study, we examined whether PFAA profiling was at least one important factor mediating central adiposity and insulin resistance, and aid in cardiovascular risk assessment in healthy Asians with normal body weight.

Methods: This was a cross-sectional study. A total of 190 healthy men (n = 87 with a mean ± SD BMI of 23.5 ± 3.5 kg/m^2) and women (n = 103 with a mean ± SD BMI of 21.4 ± 3.7 kg/m^2) residing in Singapore took part in this study. PFAA levels were measured by using an amino acid analyzer. Basic anthropometric measurements, fasting blood glucose (FBG), fasting serum insulin (FSI), and lipid profiles were obtained using standard protocols.

Results: Seven out of eighteen amino acids were significantly correlated with measures of obesity (e.g. WC, WHR, and BMI) in current participants. Among them, the plasma concentrations of five amino acids, including Phe, Tyr, Met, Ala, and His were positively associated with WHR. With the exception of His, which had no association with insulin resistance, Phe, Tyr, Met, and Ala were significantly associated with hyperinsulinemia and insulin resistance ($p < 0.05$). In contrast, no associations were observed between circulating BCAAs (i.e. Val, Leu, Ile), measures of obesity and insulin resistance. However, significant inverse associations were observed between BCAAs and TC and HDL.

Conclusions: We found that central adiposity was associated with alterations of specific amino acids. As a result, PFAAs may serve as metabolite predictors of hyperglycemia, hyperinsulinemia, and dyslipidemia in healthy participants.

Introduction

Diabetes mellitus (DM) is an increasing serious public health concern globally, which is particularly alarming in Asia (1). Compared with their Western counterparts, Asian populations are found to develop type 2 diabetes (T2D) at a lower body mass index (BMI) and at younger ages (2). One plausible reason for this interethnic difference is that Asians are more insulin resistant than Caucasians and African-Americans (3). However, the exact causes of the increased insulin resistance in Asians remain to be elucidated. The potential contributors may include the "normal-weight metabolically obese" phenotype (4), high intake of refined carbohydrates (5), and dramatically decreased physical activity levels (6).

Insulin resistance has been widely accepted to precede T2D and increase the risks for cardiovascular diseases (CVD) (7), its early detection and intervention, therefore, could be crucial to counteract the higher risks of T2D and CVD, especially in Asian populations. Recently, several studies have reported that the alterations of plasma free amino acid (PFAA) profiles were significantly associated with insulin resistant conditions (8-11). For example, the levels of branched-chain amino acids (BCAAs), i.e. valine (Val), leucine (Leu), and isoleucine (Ile), were elevated in obese subjects (12, 13). One possible etiology of BCAAs elevation in obesity is that insulin resistance decreases the utilization of amino acids and uptakes of BCAAs into muscles (14). Another potential cause is that insulin resistance reduces BCAA-catabolizing enzyme activity leading to suppression of BCAA catabolism (15). Other PFAAs, such as glutamate (Glu), serine (Ser), proline (Pro), glycine (Gly), alanine (Ala), tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp), were also changed in subjects with high visceral obesity (16). The alteration of PFAA profiles is believed to be the combined results of insulin resistance-induced accelerated protein break down in muscle and hepatic gluconeogenesis set point changes (17).

Several cohort studies have demonstrated that the alterations of PFAAs can be employed as potential biomarkers to predict the future development of T2D and CVD (18-21). However, only a few studies have examined the relationship between PFAA profiles, insulin resistance and risks for diabetes and CVD in Asian populations, who develop diabetes at a much lower BMI (1, 2). It is unclear whether the obesity-related PFAA alterations can be used as early markers for identifying diabetic risks in the healthy non-obese adults living in Singapore, a Southeast Asian country. A pioneer study by Tai et al. (22) reported that PFAA levels, including Ala, Pro, Val, Leu/Ile, Phe, Tyr, Glu/Gln, Orn, were positively associated with HOMA-IR in non-obese Chinese and Asian-Indian men. However, only males were included in the study, and the generalisability of their findings to females is not known. Previous studies have reported that the association strength of amino acid biomarkers with insulin resistance and obesity may be gender-dependent (23). Therefore, the primary goal of this study was to characterize the PFAA profiles in both healthy males and females living in Singapore and to examine the associations between PFAA profiles and CVD risk factors including dyslipidemia, hypertension, hyperglycemia, and insulin resistance. The results may have significant roles in the development of diabetes research in Singapore as well as other parts of Asia.

Material and methods

Study design and clinical measures

This study was limited to cross-sectional analyses of data from participants attending a baseline visit between June 17, 2014 and February 18, 2016. This study was a subsection of a larger study. The participants included 190 healthy adults aged 21 to 69 years: 87 males (45.8%) and 103 females (54.2%). They were recruited from the general public in

Singapore through advertisements on newspaper and posters that were placed around the National University of Singapore campus, public area and on the Clinical Nutrition Research Centre (CNRC) website. To be eligible, participants were required to be Singaporeans or individuals who have resided in Singapore for a minimum of five years, healthy males and females. Participants were excluded if they were pregnant or diagnosed with any major diseases. Prior to the study, no oral glucose tolerance tests (oGTT) were performed, but all participants were asked to restrict alcohol and caffeine-containing drinks as well as to refrain themselves from intense physical activity. All procedures involving human subjects were approved by the National Healthcare Group Domain Specific Review Board (NHG DSRB, Reference Number: 2013/00783), Singapore.

Participants arrived at the laboratory in the morning after a 10 h overnight fast. All participants gave written informed consent before starting. Two finger prick capillary blood samples were obtained for determining blood glucose concentration (FBG, mmol/L) using the HemoCue® 201+ RT Glucose analyser (HemoCue Ltd, Dronfield, UK). In addition, a total of 10 mL of venous blood was collected into Vacutainers (Becton Dickinson Diagnostics). Blood samples were separated by centrifugation at 1500 rpm for 10 min at 4 °C within 2 h of being drawn and aliquots were stored at −80 °C until analysis. Fasting serum insulin (FSI, μU/mL) was measured using the immunochemistry analyzer COBAS e411 (Roche, HITACHI, USA). Insulin resistance index HOMA-IR was calculated using FBG and FSI (HOMA-IR = FBG× FSI/22.5). Fasting lipid parameters including total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), and triglycerides (TG) were measured using chemistry analyzer COBAS c311 (Roche, HITACHI, USA). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured with an Omron blood pressure monitor (model HEM-907). The measurements were done in

duplicate and readings were averaged. The anthropometric measurements were achieved via the reported method (24, 25).

Measurement of PFAA profiles

Eighteen amino acids from plasma samples were measured using a Hitachi high-speed amino acid analyzer L-8900A system (Tokyo, Japan). The samples (600 μL) underwent protein precipitation by adding 1 mL of 5% of trichloroacetic acid (TCA) and incubated for at least 1 h at 4^{o}C. After centrifuging for 30 min at 10000 × g, the supernatant was retained and filtered by a 0.45 μm membrane filter prior to analysis. Plasma amino acids were identified using a reaction column system via the ninhydrin reaction method. The ratio of the area under the curves of each amino acid to its assigned internal standard was then plotted against a multiple point calibration curve, allowing for the quantification of the amino acid levels in plasma samples.

Statistical analysis

Baseline characteristics of the participants were presented as arithmetic means ± SDs. Linear regression models were used to examine the associations between amino acid concentrations and various clinical measures. Age, physical activity, gender, smoking status, family history of disease, and ethnicity were adjusted for in all the models. All statistical analyses were performed using Stata 11.1 (StataCorp, College Station, Tex, USA). Two sided $p < 0.05$ was considered statistically significant in all cases.

Results

Population characteristics

A total of 190 participants (87 males and 103 females) took part in this study with a mean ± SD age of 30.2 ± 11.9 years. To access physical activity, about half of the study participants

(91/190) wore an AM-180C activity monitor (Tanita, Japan) for seven consecutive days (with an average of 7163 ± 2876 steps per day). Only 1.6% of participants (3/190) smoked. Participants were more insulin resistant (80/190 were HOMA-IR ≥ 1.6), but they had a lower BMI (an average BMI of 22.4 ± 3.8 as shown in Table 1). Despite these, the participants represent generally healthy metabolic status; only 2 of them had impaired fasting glucose (IFG ≥ 5.6 mmol/L). The average TC/HDL ratio was 3.2 ± 1.0 (3.6 ± 1.2 for males and 3.0 ± 0.8 for females). Sixty-nine males (79.3%) and 97 females (94.2%) had TC/HDL ratio ≤ 4.5. The average SBP was 111.7 ± 14.3 (118.6 ± 10.0 for males and 105.8 ± 14.7 for females) and the average DBP was 66.1 ± 8.8 (68.0 ± 8.5 for males and 64.6 ± 8.8 for females). A total of 54 males (62.1%) and 94 females (91.3%) had SBP < 120 mmHg, while a total of 80 males (92.0%) and 97 females (94.2%) had DBP < 80 mmHg.

Amino acid levels in study population

Average concentrations of 18 amino acids, including 3 of BCAAs, 2 of aromatic amino acids (AAAs), 3 of sulfur-containing amino acids (SAAs), 8 of glucogenic amino acids (GAAs), and two other amino acids in healthy Singaporean adults were listed in Table 2. With the exception of taurine (Tau), aspartic acid (Asp) and Ser, Males had significanlty higher concentrations of 10 amino acids, i.e. Val, Ile, Leu, Phe, Tyr, methionine (Met), lysine (Lys), Ala, Gln, and Pro, than females.

First, we assessed whether circulating amino acid levels were linked with measures of obesity. Table 3 shows the association magnitudes (e.g. 1.40 unit increase in Val concentration was associated with 1-cm increase in WC) of amino acids with central (WC and WHR) and overall obesity (BMI). When all participants were taken together, both BCAAs and AAAs were significantly positively associated with WC, WHR, and BMI in all the unadjusted models. However, the associations between BCAAs and WC, WHR, as well as BMI did not persist after adjusting for established risk factors such as age, physical activity, gender,

187 smoking status, family history of disease, and ethnicity. In contrast, AAAs and histidine (His)
188 were positively associated with WC, WHR, and BMI even after adjusting for potential
189 confounders (Table 3). In addition, after the adjustment, both Met and Ala were positively
190 associated with WHR, whereas Gly and Ser were negatively associated with BMI.

191 *PFAA profiles related to CVD risk factors*

192 Next, we examined whether the PFAA profiles were altered in accordance with their
193 metabolic status in the current population. Table 4 shows that BCAAs were significantly
194 associated with lipid profiles, while AAAs and Ala were significantly associated with FBG
195 and HOMA-IR in the unadjusted models. After adjustment for established risk factors, seven
196 amino acids, i.e. Met, Ala, Gln, Pro, Ser, Lys, and Thr, were significantly associated with
197 FBG (Figure 1a), whereas four amino acids (Phe, Tyr, Met, and Ala) were significantly
198 associated with FSI (Figure 1b) and six amino acids (Ile, Phe, Tyr, Met, Ala, and Thr) were
199 significantly associated with HOMA-IR (Figure 1c). Furthermore, Table 5 shows that
200 BCAAs were significantly associated with TC and HDL, but Ala, Gln, and His were
201 significantly associated with TG and TG/HDL. It should be noted that none of the amino
202 acids had significant association with SBP and DBP after the adjustment (data not shown).

203 # Discussion

204 Recent metabolomics-based technologies using targeted analyses focusing on amino acids
205 and their metabolites have consistently revealed perturbation of normal amino acid
206 metabolism in obesity, insulin resistance, and T2D (23, 26, 27). Previous studies have shown
207 that the levels of BCAAs and AAAs were significantly associated with the future diagnosis of
208 DM (18) and were novel biomarkers of CVD development and thus provided early links for
209 identifying susceptibility to T2D and CVD (28). The relation of amino acid signatures with
210 insulin resistance could even be modulated by changing the dietary protein and cereal-fiber

211 contents in the absence of relevant weight loss (29). While the interethnic differences of
212 pathophysiology of insulin resistance between Asian and Caucasian populations are well
213 known (2), few studies have examined the relationships between PFAA profiles, insulin
214 resistance, and CVD risk factors in Asian population, in particular healthy Asians. The
215 population-based SABRE study by Tillin et al. (30) reported strong associations in South
216 Asian men between BCAAs and AAAs and incident diabetes. Weaker (compared to
217 European counterparts), but still significant positive associations were observed between
218 BCAAs and measures of obesity. The study by Tillin et al. is consistent with other studies
219 showing that elevated BCAAs in human obesity are associated with insulin resistance (23, 26,
220 27). The underlying mechanisms may involve the emerging role of BCAAs as potential
221 regulators of satiety, leptin, mammalian target of rapamycin (mTOR) and protein kinase C
222 (PKC) signaling (31). Our data, however, indicate that there are no significant associations
223 between BCAAs, measures of obesity, and insulin resistance. Compared to the South Asian
224 SABRE participants, the current participants were younger, had lower baseline levels of
225 glucose, insulin, and TG, as well as lower central and overall obesity. The reduced adipose
226 tissue in our participants perhaps explained the absence of associations between BCAAs,
227 measures of obesity, and HOMA-IR. Similarly, the weak correlations between BCAA levels
228 and obesity were found in another study in Indian Asians living in India (age 35 - 45) with a
229 low BMI (32). However, given that HOMA-IR was used to assess insulin resistance in ours
230 as well as previous related studies, it cannot be excluded that the lack of associations was due
231 to the indirect estimate of insulin resistance in the non-stimulated state. Recently, insulin
232 resistance assessed by using HOMA-IR was found to be unaffected by dietary interventions,
233 but the insulin resistance measured using euglycemic hyperinsulinemic clamps in the
234 maximally stimulated state was changed with all diets (29). Therefore, a plausible conclusion
235 from these observations is that the association between elevated BCAAs and insulin

resistance depends on the ethnicity, degree of obesity of the participants, and the methods used to assess insulin resistance. More research is needed to identify whether such association may exist in healthy relatively lean Southeast Asian population using an array of gold standard methods for the measurement of insulin resistance, e.g. euglycemic clamps and tracer techniques.

However, we found the elevation of 5 amino acids (Phe, Tyr, His, Met, and Ala) were significantly associated with WHR. With the exception of His, which was minimally associated with markers of insulin resistance (30), the elevations of Phe, Tyr, Met, and Ala were significantly associated with hyperinsulinemia and insulin resistance. This suggests that the alterations of specific amino acids metabolism may be the potential underlying mechanisms linking central obesity and insulin resistance. While the relationships between BCAAs and obesity-related insulin resistance were well understood (23, 26-31), the etiology of other amino acids in insulin resistant states is not fully discussed yet in literature. Increased circulating concentrations of Phe and Tyr have often been reported together with BCAAs in obese, insulin resistant, and T2DM state (9, 12, 13). Phe and Tyr are metabolized to catecholamines, which could be an important factor for the development of central obesity and/or vice versa (33). Our data indicate that AAAs (Phe and Tyr) and related metabolites are associated with insulin resistance even in the absence of obesity, suggesting that dysregulation of AAAs metabolism may be an early indication of the progression to insulin resistance and T2D.

Previously, a number of groups reported the increased circulating concentrations of Met and its catabolic derivative cysteine (Cys) in obesity, insulin resistance, and T2D (34, 35). The current study extends these findings to young healthy Asian population predominantly without obesity. Our results show significant associations between circulating Met, fasting hyperglycemia and hyperinsulinemia, indicating high level of Met was inversely associated

261 with insulin sensitivity. This is consistent with previous study showing that dietary Met
262 restriction enhances peripheral insulin sensitivity (36). The potential underlying mechanism
263 is that Met restriction changes adiposity, releases insulin-sensitizing hormone, adiponectin,
264 from adipose tissue, and improves the liver function. Our data supports the notion that
265 increased Met levels in plasma resulting from central adiposity may contribute to
266 hyperinsulinemia and insulin resistance.

267 In addition to Met, there were positive correlations between circulating Ala and fasting
268 hyperglycemia and hyperinsulinemia. As a NEAA, Ala is metabolized to pyruvate and is
269 derived from BCAAs via glucose-alanine cycle (37). Therefore, Ala is involved in
270 maintaining glucose homeostasis. Previously, Nakamura et al. (38) found that Ala
271 concentration was significantly correlated with BMI and insulin related variables such as C-
272 peptide, insulin, HOMA-IR, and adiponectin, in T2D patients. In line with this finding,
273 another study also reported significant associations between Ala and HOMA-IR in healthy
274 Chinese and Asian-Indian men (22). Our data indicate that Ala is positively correlated with
275 not only FBG, FSI, and HOMA-IR but also WHR, suggesting that this Ala-insulin resistance
276 relationship may result from protein turnover in association with increased central obesity.
277 Another possible explanation is that the central obesity-induced increment of Ala is probably
278 due to reduced entry of glucose into mitochondria for full oxidation, which also alters
279 BCAAs metabolism generating insulin resistance.

280 While the effect of PFAAs on obesity and insulin resistance has been extensively investigated,
281 the effect on cholesterol metabolism, which is also associated with metabolic syndrome, has
282 not been studied in detail. The BCAA Leu is known as a precursor of cholesterol and serves
283 as a potent modulator of cholesterol metabolism. It was found that Leu supplement decreased
284 TC and LDL levels by 27% and 53%, respectively, in mice on high-fat (60% fat calories) diet
285 (39). Another study by Torres-Leal et al. (40) reported that Leu attenuated the cholesterol

levels in rats treated with high-fat diet. The reduction in cholesterol levels was found to be independent of changes in body weight. The underlying molecular mechanisms are largely related to mTOR signalling activation (39). Although many studies have reported cholesterol-reducing effects of Leu in animal models, Newgard et al. (13) observed that plasma Leu levels significantly increased in obese participants with hyperlipidemia compared with non-obese controls. Few studies have been undertaken to examine the cholesterol-reducing effects of Leu in non-obese populations. Although only 24 participants (12.6%) in our study cohort have a TC levels higher than 6.2 mmol/L (240 mg/dL), our data have shown that Leu is adversely correlated with TC, and suggest that dysregulation of Leu metabolism may be an early event in the progression to hypercholesterolemia. On the other hand, cholesterol-lowering effects of Val and Ile were also reported by others (41), but the underlying mechanisms remained elusive. Further elucidation of BCAAs signalling, via mTOR or other potential mediators, will be required to discover the relevant mechanisms underlying the increase in BCAA levels in body cholesterol metabolism. Finally, although TC > 6.2 mmol/L is recognized as a risk factor for CVD, the risk is lower if a high proportion is made up of the protective HDL than if the elevation is due primarily to increased amounts of LDL. Table 5 shows significant inverse associations between BCAAs, TC, and HDL, suggesting that the beneficial effects of BCAAs on TC could be due to the attenuated HDL levels, which is not desirable.

Conclusions

Early intervention against conventional T2D and CVD risk factors, such as hyperglycemia, hyperinsulinemia, hypertension, and dyslipidemia, is crucial to prevent the overt diseases onset. Our results suggest that the information generated from PFAA profiles is useful in identifying healthy, relatively non-obese individuals who are at high risk of developing

insulin resistance and/or dyslipidemia. Our data also support the hypothesis that either central obesity is a risk factor for altered four plasma amino acid levels, including Phe, Tyr, Ala, and Met, or PFAAs alteration is a risk factor for central obesity. Regardless of the outcomes, the current study has shown that the combinations of four amino acids are significantly associated with increased insulin resistance in healthy Singaporean adults. The intriguing result of the present study is that there is no relationship between BCAAs and insulin resistance that warrants further investigation. Our study, however, indicates that BCAAs could help to improve cholesterol metabolism.

Our study has several limitations. First, this is a cross sectional study and thus causal inferences cannot be drawn. Second, the sample size of this study is not large enough. Future studies should be conducted with larger sample sizes. Third, although HOMA-IR has become a widely used clinical and epidemiological tool, it is not a direct measurement of insulin resistance. Therefore, the use of HOMA-IR to assess insulin resistance may have potential problems and fails to show a close relationship with whole body insulin resistance assessed by euglycemic clamp techniques (29). Finally, dietary effects on amino acid profiles and insulin resistance were observed in some previous reports (29, 42), but other studies yielded contrary results showing that increased protein intake was not observed as evidence for higher BCAA levels (18, 22). Moreover, the pool size of free BCAAs is quite small and constant because of the continuous supply from muscle (43). Although we did not evaluate dietary nutrient intake, the circulating BCAAs and other EAAs are unlikely dominantly regulated by dietary intake, at least in the steady state of the present study. Despite these limitations, the results of our study provide evidence of the linkage between alterations of PFAAs, central adiposity, and increased insulin resistance in Asian populations.

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