Serum Glycerophosphate Levels are Increased in Japanese Men with Type 2 Diabetes

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Abstract

Objective To identify metabolites showing changes in serum levels among Japanese male with diabetes.

Methods We performed metabolite profiling by coupling capillary electrophoresis with electrospray ionization time-of-flight mass spectrometry using fasting serum samples from Japanese male subjects with diabetes (n=17), impaired glucose tolerance (IGT; n=5) and normal glucose tolerance (NGT; n=14).

Results Other than the expected differences in characteristics related to abnormal glucose metabolism, the percent body fat was significantly different among subjects with diabetes, IGT and NGT (27.3±6.2, 22.2±4.5 and 19.2±6.0%, respectively, p=0.0022). Therefore, percent body fat was considered as a possible confounding factor in subsequent analyses. Of 560 metabolites detected using our platform, the levels of 74 metabolites were quantified in all of the serum samples. Significant differences between diabetes and NGT were observed for 24 metabolites. The top-ranked metabolite was glycerol-3-phosphate (glycerophosphate), which was significantly higher in subjects with diabetes than in those with NGT, even after Bonferroni correction for multiple testing (11.7±3.6 vs. 6.4±1.9 μM, respectively; corrected p=0.0222). Stepwise multiple regression analyses revealed that serum glycerophosphate levels were significantly correlated with 2-h plasma glucose after a 75-g oral glucose tolerance test (r=0.553, p=0.0005), independently of other characteristics, including FPG and HbA1c.

Conclusion Serum glycerophosphate levels were found to be elevated in Japanese men with diabetes, and correlated with 2-h PG, independent of FPG and HbA1c. Namely, serum glycerophosphate level at fasting condition can be a marker for predicting glucose intolerance. These results warrant further studies to evaluate the relevance of glycerophosphate in the pathophysiology of diabetes.

Key words: glycerophosphate, diabetes, metabolite profiling, gluconeogenesis, postprandial hyperglycemia

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Introduction

Type 2 diabetes mellitus is a heterogeneous disorder of glucose metabolism characterized by insulin resistance and pancreatic β-cell dysfunction (1-4). As a result, diabetes is associated with marked changes in many metabolites, which are not necessarily related to plasma glucose levels, but may

be related to insulin resistance, decreased serum insulin levels and/or other conditions often accompanying diabetes, such as obesity. Identifying such metabolites may lead to the establishment of new markers for abnormal glucose tolerance or diabetes, and the associated pathophysiological conditions. Metabolite profiling studies have already shown that fatty acids (FAs), amino acids (AAs), including branched-chain AAs, phosphatidylcholines (PCs) and α-

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Table 1. Differences in Clinical Characteristics among the Study Groups

Characteristic	Diabetes	IGT	NGT	p	
				Diabetes vs. NGT	Among all 3 groups
Number	17	5	14	-	-
Age (yr)	61.7±12.3	58.0±9.9	65.0±13.6	0.4856	0.5394
Height (cm)	161.8±5.7	159.6±5.8	164.3±6.4	0.2524	0.2802
Body weight (kg)	68.5±9.5	58.4±12.8	65.5±10.8	0.4048	0.1751
Body mass index (kg/m ²)	26.1±2.9	22.7±3.6	24.3±3.9	0.1366	0.1088
Percent body fat (%)	27.3±6.2	22.2±4.5	19.2±6.0	0.0009**	0.0022**
Waist circumference (cm)	87.2±7.2	85.0±9.7	86.4±9.4	0.7800	0.8686
Hip circumference (cm)	95.2±6.4	93.5±5.5	96.1±7.7	0.7172	0.7441
Waist/Hip ratio	0.916±0.047	0.907±0.068	0.899±0.056	0.3617	0.6768
Fasting plasma glucose (mg/dL)	157.9±49.6	93.6±12.0	90.9±7.3	<0.0001**	<0.0001**
2h plasma glucose (mg/dL)	312.2±93.5	164.8±15.2	102.6±15.7	<0.0001**	<0.0001**
HbA1c (%)	7.59±1.19	5.44±0.18	5.28±0.21	<0.0001**	<0.0001**
Systolic blood pressure (mmHg)	133.9±17.6	136.0±7.3	131.9±20.1	0.7673	0.8942
Diastolic blood pressure (mmHg)	77.8±12.7	82.4±4.3	80.4±12.3	0.5699	0.6993
Total cholesterol (mg/dL)	218.6±38.9	190.8±41.8	193.2±41.0	0.0874	0.1648
Triglyceride (mg/dL)	199.8±161.6	105.4±40.2	148.8±155.5	0.3813	0.4026
HDL cholesterol (mg/dL)	47.5±11.2	49.0±10.6	52.6±11.1	0.7121	0.6623

*p<0.05 and **p<0.01. Data are means±SD. Differences were analyzed using ANOVA.

hydroxybutyrate are associated with glucose intolerance or insulin resistance (5-10).

In general, insulin resistance occurs before the development of diabetes, with a compensatory increase in insulin secretion to maintain plasma glucose levels within the normal range. Glucose intolerance becomes evident as this compensatory increase in insulin secretion declines. Notably, Japanese subjects have been reported to show a weaker compensatory increase in insulin secretion, and are more likely to develop diabetes without developing marked insulin resistance, as compared with Caucasian individuals (11-13). To date, the majority of these metabolite profiling studies were conducted with Caucasian subjects. Therefore, considering the difference in the etiology of diabetes between Japanese and Caucasian individuals, it is feasible that the metabolites related to glucose intolerance or diabetes in Japanese individuals may be different from those in Caucasian individuals.

Therefore, in this study, we conducted metabolite profiling by coupling capillary electrophoresis with electrospray ionization time-of-flight mass spectrometry (CE-TOFMS) to identify metabolites showing changes in Japanese subjects with diabetes or impaired glucose tolerance (IGT) compared with individuals with normal glucose tolerance (NGT).

Materials and Methods

Subjects

Male subjects with diabetes (n=17), and their age and

sex-matched non-diabetic subjects (n=19) were randomly selected from among the participants of the Funagata study (from those who attended in 2000, 2001 and 2002), a Japanese population-based study conducted in an agricultural area located about 400 km north of Tokyo. The design of the study has been reported in more detail elsewhere (14). The non-diabetic subjects were further divided into 2 groups, subjects with IGT (n=5) and NGT (n=14).

The present study was approved by the Ethics Committee of the Yamagata University School of Medicine, and informed consent was obtained from all of the participants. The value for HbA1c (%) is estimated as an National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula $\text{HbA1c (\%)} = \text{HbA1c (Japan Diabetes Society:JDS)(\%)} + 0.4\%$, considering the relational expression of HbA1c (JDS)(%) measured by the previous Japanese standard substance and measurement methods and HbA1c (NGSP) (15). The clinical characteristics of the study groups are shown in Table 1. Glucose tolerance was evaluated by a 75-g oral glucose tolerance test (OGTT) according to the 1998 World Health Organization criteria (16). The serum samples collected simultaneously with the samples for fasting plasma glucose (FPG) for OGTT were used for metabolite profiling. Subjects with known diabetes before the examination were excluded; therefore, only those subjects diagnosed with diabetes for the first time were included in this study as having diabetes.

Metabolite profiling

Metabolite profiling was conducted as previously de-

scribed (17, 18). In brief, after a 10-h overnight fast, blood samples were collected from the subjects by phlebotomy between 07:00 and 10:00 hours. Serum samples were immediately prepared and stored at -20°C until used. We performed CE-TOFMS using the serum samples to identify metabolites showing differences among subjects with diabetes, IGT and NGT. To facilitate peak identification and quantification, 560 reference metabolites listed in the KEGG LIGAND database were analyzed before testing the serum samples. Global mass scanning over a 50-1,000 m/z range was conducted in the CE-TOFMS mode.

Statistical analysis

Data are presented as means $\pm$ standard deviation (SD). The statistical significance of differences in clinical characteristics values between subjects with diabetes and NGT was assessed using Student's *t*-test. Differences among the three groups were evaluated by analysis of variance (ANOVA). Differences in serum metabolite levels were assessed by ANOVA with adjustment for age and percent body fat. Scheffe's *post hoc* test was used after ANOVA. Univariate and stepwise multivariate linear regression analyses were performed to determine associations between clinical characteristics and glycerol-3-phosphate (glycerophosphate) levels. Spearman's rank correlation coefficients were also used to measure the correlations between clinical characteristics and glycerophosphate levels. In analyses, the serum glycerophosphate levels were log-transformed ($\log_{10}$) to approximate a normal distribution. Values of $p < 0.05$ were accepted as significant. All analyses were conducted using Stat View for Macintosh, version 5.0 (SAS Institute Inc., Cary, NC).

Results

Clinical characteristics of the study subjects

The clinical characteristics of the study subjects are shown in Table 1. In addition to expected differences in characteristics related to abnormal glucose metabolism, we found that the percent body fat was also significantly different among subjects with diabetes, IGT and NGT (27.3 ± 6.2 , 22.2 ± 4.5 and $19.2 \pm 6.0\%$, respectively; $p = 0.0022$). In other words, percent body fat increased significantly from NGT to IGT to diabetes. Therefore, percent body fat was included as a possible confounding factor in subsequent analyses.

Serum glycerophosphate levels were higher in subjects with diabetes

Of 560 metabolites monitored by our platform, the levels of 74 metabolites were quantified by the CE-TOFMS analyses in all of the serum samples. Significant differences between the subjects with diabetes and NGT were detected for 24 metabolites (Table 2). Most of the metabolites showing differences between the two groups were AAs or FAs, and were significantly higher in subjects with diabetes. Serum levels of glycerophosphate and glycerophosphorylcholine

were also significantly higher in subjects with diabetes. Since 74 metabolites were measured simultaneously and were used in the analyses, adjustment for multiple testing was deemed essential. After adjustment for multiple testing with Bonferroni correction, only glycerophosphate levels remained significantly higher in subjects with diabetes compared with subjects with NGT (11.7 ± 3.6 vs. 6.4 ± 1.9 μM , respectively; corrected $p = 0.0222$).

Glycerophosphate is a key molecule involved in gluconeogenesis, a process that is upregulated in diabetes. Therefore, the serum levels of the metabolites involved in the gluconeogenic pathway among the three groups of subjects are summarized in Fig. 1, in relation to this pathway. The gluconeogenic AAs glycine, serine, and alanine were increased in subjects with diabetes, but glycerol, a gluconeogenic lipid, was not. Glycerophosphorylcholine, a precursor of glycerophosphate, was also increased in subjects with diabetes.

Independent correlations between postprandial glucose levels and serum glycerophosphate levels

Simple regression analyses revealed significant correlations between most of the clinical characteristics with serum glycerophosphate levels. However, 2-h plasma glucose (2-h PG) after the OGTT gave the highest correlation coefficient ($r = 0.553$, $p = 0.0005$) (Table 3). Spearman's rank correlation (non-parametric) analyses also revealed the highest significant correlation between 2-h PG and serum glycerophosphate levels ($r = 0.693$, $p < 0.0001$) (Table 3). We next performed stepwise multivariate regression to identify characteristics showing independent associations with serum glycerophosphate levels. In this analysis, only 2-h PG remained significantly and independently correlated with serum glycerophosphate levels.

Discussion

Percent body fat adjusted analyses showed that serum glycerophosphate levels were significantly higher in subjects with diabetes than in those with NGT. This difference showed the greatest statistical significance of all of the metabolites tested in this study ($p = 0.0003$), and this remained true after the Bonferroni correction for multiple testing. Therefore, these results indicate that serum glycerophosphate levels increased with the progression of glucose intolerance from NGT to diabetes.

We next examined the correlations between serum glycerophosphate levels and diabetes. To achieve this, we first evaluated the correlations of serum glycerophosphate levels with clinical characteristics examined in all subjects. Univariate regression analyses revealed significant correlations between serum glycerophosphate levels and many clinical characteristics. However, multiple stepwise regression analyses revealed that most of the correlations, except for 2-h PG, were not independent. Therefore, only 2-h PG was independently correlated with serum glycerophosphate levels.

Table 2. Differences in Metabolites between Subjects with Diabetes and NGT

Metabolite	Higher in	p value		Metabolite	Higher in	p value	
		Crude	Bonferroni			Crude	Bonferroni
Glycerophosphate	DM	0.0003**	0.0222*	Cystine	DM	0.1688	1
Octanoate	DM	0.0017**	0.1258	Trimethylamine N-oxide	NGT	0.1708	1
Glycerophosphorylcholine	DM	0.0038**	0.2812	Sarcosine	DM	0.1714	1
Thr	DM	0.0045**	0.333	Succinate	NGT	0.1735	1
Arg	DM	0.0068**	0.5032	Pipecolate	DM	0.1832	1
Phe	DM	0.0074**	0.5476	N,N-Dimethylglycine	DM	0.1856	1
Methionine sulfoxide	DM	0.0076**	0.5624	Uridine	NGT	0.2068	1
Hexanoate	DM	0.0080**	0.5920	beta-Ala	DM	0.2131	1
Tyr	DM	0.0107*	0.7918	Creatine	DM	0.2185	1
Heptanoate	DM	0.0110*	0.8140	5-Oxoproline	NGT	0.2793	1
Ser	DM	0.0116*	0.8584	Malate	NGT	0.3046	1
His	DM	0.0135*	0.9990	Citrulline	NGT	0.3125	1
2-Aminobutanoate	DM	0.0157*	1	Betaine	NGT	0.3229	1
Acetohydroxamate	NGT	0.0159*	1	Creatinine	DM	0.3268	1
Lactate	NGT	0.0178*	1	5-Oxoproline	NGT	0.3307	1
Leu	DM	0.0198*	1	Glutarate	DM	0.4093	1
Choline	DM	0.0203*	1	3-Phenylpropionate	DM	0.4274	1
Pro	DM	0.0203*	1	Gly	DM	0.4287	1
Asn	DM	0.0204*	1	Benzoate	DM	0.4382	1
Lys	DM	0.0262*	1	Citrate	NGT	0.4476	1
Ala	DM	0.0296*	1	2-Hydroxybutyrate	DM	0.4968	1
Hypoxanthine	NGT	0.0376*	1	Carnitine	NGT	0.4999	1
Taurine	NGT	0.0416*	1	4-Methylbenzoate	DM	0.5060	1
Ornithine	DM	0.0432*	1	Pentanoate	NGT	0.5091	1
Ile	DM	0.0627	1	Isethionate	NGT	0.5742	1
Urea	DM	0.0629	1	Glu	DM	0.6995	1
Trp	DM	0.0634	1	Glycerate	NGT	0.7374	1
Allantoin	NGT	0.0640	1	gamma-Butyrobetaine	NGT	0.7559	1
Val	DM	0.0640	1	Guanidinoacetate	NGT	0.8391	1
Gln	DM	0.0641	1	Butanoate	DM	0.8612	1
Azelate	NGT	0.0773	1	Glucosamine	NGT	0.8986	1
2-Hydroxyglutarate	DM	0.0862	1	cis-Aconitate	NGT	0.9299	1
Asp	DM	0.1032	1	3-Methylbutanoate	DM	0.9357	1
Hydroxyproline	DM	0.1080	1	2-Hydroxypentanoate	DM	0.9370	1
Thymine	DM	0.1092	1	3-Hydroxybutyrate	NGT	0.9571	1
3-Methylhistidine	DM	0.1151	1	N-Acetyl-beta-alanine	NGT	0.9672	1
Z-Gly-Pro	DM	0.1156	1	Urate	NGT	0.9898	1

Data were compared using ANOVA with adjustment for age and percent body fat. * $p < 0.05$ and ** $p < 0.01$.

Therefore, serum glycerophosphate levels seem to be a reliable marker for increased 2-h PG, which does, at least, in part, correspond to postprandial hyperglycemia, and 2-h PG is acknowledged to be a stronger risk factor for diabetes-related macrovascular complications than elevated FPG (19, 20).

The mechanisms involved in the increase in serum glycerophosphate levels observed in diabetes are not clearly explained, since various mechanisms including impaired insulin secretion and insulin resistance, and their subsequent

consequences such as increased gluconeogenesis, increased glycogenolysis, and increased fatty acids synthesis together can explain the fact. Nevertheless, since glycerophosphate is an intermediate metabolite of gluconeogenesis, which is induced in diabetes (21), an increased gluconeogenesis may be an explanation of the fact, although this explanation remains to be evaluated. Further, glycerophosphate does not permeate the cell membrane freely, and, thus, serum glycerophosphate levels may not reflect the phenomenon occurred in cells. However, we speculate that serum glycerophosphate

Figure 1. Boxplots showing the serum levels of metabolites involved in gluconeogenesis. Differences in the serum levels of the metabolites were assessed by ANOVA with adjustment for age and percent body fat. Scheffe's *post hoc* test was used after ANOVA. DM: diabetes, IGT: impaired glucose tolerance, NGT: normal glucose tolerance, PG: plasma glucose

levels reflect, at least, a part of their levels in cells.

As the gluconeogenic AAs, glycine, serine and alanine were found as increased in subjects with diabetes in the study (Fig. 1), levels of metabolites involved in gluconeogenesis are generally increased in diabetes. Therefore, it could be expected that serum glycerophosphate levels are increased in diabetes, since glycerophosphate is a key gluconeogenic molecule. However, to date, no studies have reported associations between serum glycerophosphate levels and diabetes or the characteristics related to glucose intolerance. Therefore, the present findings are particularly novel. This may be because the above-mentioned metabolite profiling studies did not seem to assess glycerophosphate (5-10). Therefore, it is likely that serum glycerophosphate levels would be increased also in Caucasian subjects, although this will need to be tested. We believe that the increase in serum glycerophosphate levels in diabetes would be a general phenomenon, independent of ethnicity. Further studies are needed to confirm the association between serum glycerophosphate levels and diabetes in other ethnicities including Caucasians.

Glycerophosphorylcholine is converted to glycerophosphate by the enzyme glycerophosphocholine phosphodiesterase (EC 3.1.4.2), and it is a glycerophosphate precursor that was found to be increased in subjects with diabetes (Table 2, Fig. 1). This increase can be explained as a consequence of the gluconeogenic shift in lipid metabolism in diabetes. Another gluconeogenic lipid, glycerol, was not increased in diabetes, which is consistent with another metabolite profiling study (9). Thus, the increase in serum glycerophosphorylcholine levels may have a greater impact on gluconeogenesis than does the change in serum glycerol

levels in diabetes, at least in the fasting state. However, the mechanisms involved in the increase are not fully clear. A study with Caucasian subjects showed decreased serum concentrations of glycerophosphorylcholine, lysophosphatidylcholines, and phosphatidylcholines in diabetes (10). In a study of diabetic mice, the plasma concentrations of lysophosphatidylcholines, precursors of glycerophosphorylcholine, were decreased, whereas the plasma concentrations of phosphatidylcholines, which are also derived from lysophosphatidylcholines, were increased (22). The authors of that study postulated that diabetes promotes a shift from lysophosphatidylcholine to phosphatidylcholine levels (22). Since the serum glycerophosphorylcholine levels were increased in subjects with diabetes in the present study, a shift from lysophosphatidylcholine to glycerophosphorylcholine levels or upregulation of the metabolic pathway from phosphatidylcholine to lysophosphatidylcholine to glycerophosphorylcholine to glycerophosphate in diabetes seems to offer an alternative explanation for these findings, which should be clarified in future studies.

The serum levels of several AAs and FAs were significantly increased in subjects with diabetes. These findings were generally consistent with the results of previous studies of subjects with diabetes-related conditions such as glucose intolerance, obesity or insulin resistance (5-10). AAs, including branched-chain and neutral AAs, and FAs have been reported to be increased in such conditions (5-10). However, regarding the gluconeogenic AAs glycine, serine and alanine, these were increased in subjects with diabetes in the present study, but some inconsistencies have been reported (6-10). In a study of obese subjects, the serum alanine levels were significantly increased in obese subjects,

Table 3. Correlations between Clinical Characteristics and Serum Glycerophosphate Levels

Characteristic	Simple		Stepwise	
	Non-parametric	Parametric	Model 1	Model 2
r^2	-	-	0.341	0.430
Age (yr)	-0.074	-0.112**	NA	NA
Height (cm)	-0.101	-0.209*	-	NA
Body weight (kg)	0.222	0.172**	-	NA
Body mass index (BMI) (kg/m ²)	0.308	0.301**	NA	NA
Percent body fat (%)	0.440**	0.402**	NA	NA
Waist circumference (cm)	0.171	0.155*	NA	NA
Hip circumference (cm)	0.171	0.142	-	-
Waist/Hip ratio	0.08	-0.108	-	-
Fasting plasma glucose (mg/dL)	0.687**	0.428**	-	NA
2hr plasma glucose (mg/dL)	0.693**	0.553**	0.553	0.553
HbA1c (%)	0.637**	0.531**	-	NA
Systolic blood pressure (mmHg)	0.131	0.145**	NA	NA
Diastolic blood pressure (mmHg)	-0.001	0.065**	-	NA
Total cholesterol (mg/dL)	0.304	0.236**	NA	NA
Triglyceride (mg/dL)	0.285	0.145**	-	NA
HDL cholesterol (mg/dL)	-0.059	-0.008**	-	NA

Spearman's rank correlation coefficients (non-parametric), and correlation coefficients from univariate and stepwise multivariate regression analyses (parametric) are shown. Serum glycerophosphate levels were log-transformed (log10) to approximate a normal distribution. * $p < 0.05$ and ** $p < 0.01$. -, not included in the model; NA, not accepted as significant in stepwise multivariate regression analysis. Model 1: adjusted for age, body mass index, percent body fat, waist, 2-h plasma glucose, systolic blood pressure, and total cholesterol. Model 2: adjusted for all characteristics correlated with serum glycerophosphate levels in univariate analyses.

whereas the serum glycine levels were significantly decreased (6). On the other hand, in nondiabetic subjects, the serum glycine levels were positively correlated with insulin resistance, with the third-ranked strongest correlation (8). The subjects in these studies showed some features associated with diabetes, but did not have overt diabetes, which may explain the differences in results. Ethnic differences may also explain the discrepancies between these studies. Interestingly, all of these gluconeogenic AAs were decreased in diabetic *db/db* mice. The authors of that report explained their results as a consequence of the increased uptake of these AAs to meet the demand of increased gluconeogenesis. However, it remains to be seen whether this explanation also applies for people with diabetes or is specific to the diabetic model used in that study.

The limitations of the present study need to be mentioned. First, the number of subjects included in this study was not substantial, which reduced the statistical power of the analysis, particularly when coupled with the necessary adjustments for multiple testing. The statistical power estimated using Sampsize software (<http://sampsize.sourceforge.net/iface/index.html>) to determine the difference in serum octananoate, the second highest ranked metabolite to be associated with diabetes, was about 76% to detect a mean difference of 100 μ M at a level of significance of 0.05 after Bonferroni correction, whereas the power for serum Ala levels was about 47% to detect a mean difference of 190 μ M at

the same level of significance. Therefore, because of the low statistical power, the differences in 23 metabolites between the diabetes and NGT groups were significant in crude analyses, but not after Bonferroni correction. Had we included a larger number of subjects, it is likely that some of these metabolites would show significant differences between the two groups. The limited number of the subjects may also increase the potential for selection bias. However, since we randomly selected the subjects with diabetes and the control subjects, such selection bias may not be so substantial to confuse the results. Second, to eliminate possible sex differences, we only included males. However, this was important because men generally show more pronounced insulin resistance compared with women. Accordingly, inclusion of women might have weakened the associations. Thus, studies with more subjects, as well as studies in women, are warranted to confirm and extend our current results.

In conclusion, serum glycerophosphate levels were elevated in Japanese men with diabetes compared with those in subjects with NGT. The serum glycerophosphate levels were correlated with 2-h PG, independent of other clinical characteristics such as FPG and HbA1c. Namely, the serum glycerophosphate level in fasting condition can be a useful marker for predicting 2-h PG, which is generally accepted as a marker for abnormal glucose metabolism. These results warrant further studies to determine whether this association is common to all subjects with diabetes, or specific to Japa-

nese subjects, and to evaluate the relevance of glycerophosphate in the pathophysiology of diabetes.

The authors state that they have no Conflict of Interest (COI).

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