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Metabolic Profiling of Plasma in Overweight/Obese and Lean Men using Ultra Performance Liquid Chromatography and Q-TOF Mass Spectrometry (UPLC−Q-TOF MS)

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Obesity is currently epidemic in many countries worldwide and is strongly related to diabetes and cardiovascular disease. This study investigated the differences in metabolomic profiling between overweight/obese and normal-weight men. Overweight/obese ($n = 30$) and age-matched, normal-weight men ($n = 30$) were included. Anthropometric parameters, conventional metabolites, and biomarkers were measured. Metabolomic profiling was analyzed with UPLC−Q-TOF MS. Overweight/obese men showed higher levels of HOMA-IR, triglycerides, total cholesterol, and LDL-cholesterol, and lower levels of HDL-cholesterol and adiponectin than lean men. Overweight/obese men showed higher proportion of stearic acid and lower proportion of oleic acid in serum phospholipids. Additionally, overweight/obese individuals showed higher fat intake and lower ratio of polyunsaturated fatty acids to saturated fatty acids. We identified three lyso-phosphatidylcholine (lysoPC) as potential plasma markers and confirmed eight known metabolites for overweight/obesity men. Especially, overweight/obese subjects showed higher levels of lysoPC C14:0 and lysoPC C18:0 and lower levels of lysoPC C18:1 than lean subjects. Results confirmed abnormal metabolism of two branched-chain amino acids, two aromatic amino acids, and fatty acid synthesis and oxidation in overweight/obese men. Additionally, the amount of dietary saturated fat may influence the proportion of saturated fatty acids in serum phospholipids and the degree of saturation of the constituent acyl group of plasma lysoPC.

Keywords: metabolic profiles • obesity • UPLC−Q-TOF MS • lyso-phosphatidylcholine • saturated fat

Introduction

Obesity has reached epidemic proportions in many countries around the world and is strongly related to diabetes and cardiovascular disease.[1] Whereas many comparisons of obese and lean subjects exist in the literature, they often focus on a small group of experimental variables. Obesity cosegregates with metabolic abnormalities, including dyslipidemia and glucose intolerance;[2] however, obesity-induced perturbations in metabolism have not been clearly established. Complex etiologies interacting with environmental factors highlight the need to understand how metabolite profiles are altered in this state.

Metabolomics is an important technological discipline that focuses on the measurement of the relative concentrations of endogenous small molecules in biofluids, which characterizes changes in metabolism[3] and helps unravel the metabolic state of biological systems.[4] Metabolomics involves establishing relationships between phenotype and metabolism, which are key aspects of biological function. These approaches have been applied to identify serum/plasma metabolic markers involved in obesity,[5−7] diabetes,[8−10] and coronary artery disease[11] through animal models or in humans.

A new method of ultraperformance liquid chromatography coupled with Q-TOF mass spectrometry (UPLC−Q-TOF MS) was developed for sample analysis to obtain better quality and throughput for the analysis of complex mixtures. This sensitive, high-resolution system can acquire multiparametric metabolite profiles from biofluids rapidly and effectively as a powerful

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metabolomics tool.[12,13] Here, we employed metabolomics analyses based on UPLC−Q-TOF MS to gain a broader understanding of metabolic differences between overweight/obese and lean men.

Materials and Methods

Subjects. Study subjects were 60 healthy men between 30 and 50 years of age. They were recruited during routine checkups at a health promotion center at National Health Insurance Corporation Ilsan Hospital. Overweight/obese men were defined as those with a body mass index (BMI) greater than or equal to 25 kg/m^2. Finally, 30 overweight/obese subjects ($27 \leq$ BMI ≤ 33) and 30 age-matched, lean subjects as controls ($18 \leq$ BMI ≤ 23) were included in this study. Subjects were excluded if they had significant cardiopulmonary, renal, or liver disease, or were taking a diabetes medication, systemic corticosteroids, or using weight loss mediation. The study protocol was approved by the Institutional Review Board of Yonsei University, and all participants provided written informed consent.

Anthropometric Parameters, Blood Pressure, and Blood Collection. Body weights and heights were measured in unclothed subjects in the morning for the calculation of BMI (kg/m^2). Waist and hip circumferences were measured for the calculation of waist/hip ratio (WHR). Blood pressures (BPs) were measured in the left arm of seated patients with an automatic BP monitor (TM-2654, A&D, Tokyo, Japan) after a 20-min rest. After a 12-h fasting period, venous blood specimens were collected in EDTA-treated and plain tubes, centrifuged to yield plasma or serum, and stored at −70 °C until analysis.

Assessment of Dietary Intake. The dietary intake was assessed with a 24-h recall method and semiquantitative food frequency questionnaire. Dietary energy values and nutrient content were calculated using the Computer Aided Nutritional analysis program (CAN-pro 2.0. Korean Nutrition Society, Seoul, Korea). Total energy expenditure (TEE) was calculated from activity patterns including basal metabolic rate (BMR), physical activity for 24-h and specific dynamic action of food. BMR of each subject was calculated with the Harris-Benedict equation.

Glucose, Insulin, and HOMA-IR. Fasting glucose levels were measured using a glucose oxidase method with a Beckman Glucose Analyzer (Beckman Instruments, Irvine, CA). Insulin levels were measured by radioimmunoassay using commercial kits from Immuno Nucleo Corporation (Stillwater, MN). Insulin resistance (IR) was calculated by the homeostasis model assessment (HOMA) using the following equation: IR = [fasting insulin (μIU/mL) × fasting glucose (mmol/L)]/22.5.

Serum Lipid Profile. Fasting total cholesterol and triglycerides were measured using commercially available kits on a Hitachi 7150 Autoanalyzer (Hitachi Ltd., Tokyo, Japan). After precipitation of serum chylomicrometers using dextran sulfate magnesium, the concentrations of LDL-cholesterol and HDL-cholesterol in the supernatants were measured using an enzymatic method. LDL-cholesterol was indirectly estimated in subjects with serum triglyceride concentrations less than 400 mg/mL using the Friedewald formula {LDL-cholesterol = total-cholesterol − [HDL-cholesterol + (triglycerides/5)].

Plasma Lp-PLA$_2$ Activity, Oxidized LDL, and Adiponectin Concentrations. The activity of Lp-PLA$_2$ (phospholipase A$_2$), which is also known as platelet-activating factor acetylhydrolase (PAF-AH), was measured using a previously described modified method.[14] Plasma oxidized LDL (ox-LDL) was measured using an enzyme immunoassay (Mercodia, Uppsala, Sweden), and the resulting color reaction was read at 450 nm on a Victor^2 plate reader (Perkin-Elmer Life Sciences, Turku, Finland). Plasma adiponectin concentrations were measured using an enzyme immunoassay (Human Adiponectin ELISA kit, B-Bridge International Inc., CA, USA). Assays were read using a Victor^2 plate reader at 450 nm, and wavelength correction was set to 540 nm.

Lipid Peroxidation: Urinary 8-Epi-prostaglandin F$_{2\alpha}$ and Plasma Malondialdehyde. Urine was collected in polyethylene bottles containing 1% butylated hydroxytoluene after 12 h of fasting. The tubes were immediately covered with aluminum foil and stored at −70 °C until analysis. The compound 8-epi-prostaglandin F$_{2\alpha}$ (8-epi-PGF$_{2\alpha}$) was measured using an enzyme immunoassay (BIOXYTECH urinary 8-epi-PGF$_{2\alpha}$ Assay kit, OXIS International Inc., OR). The resulting color reaction from the enzyme immunoassay was read at 650 nm on a Victor^2 plate reader. Urinary creatinine was determined by the alkaline picrated (Jeffe) reaction. Urinary 8-epi-PGF$_{2\alpha}$ concentrations were expressed as pmol/mmol creatinine. Malondialdehyde (MDA) was measured from thiobarbituric acid-reactive substances (TBARS Assay Kit, Zepto-Metrix Co., Buffalo, NY). Assays were read using a Victor^2 plate reader at 540 nm.

Serum hs-CRP. Serum hs-CRP (C-reactive protein) concentrations were measured with an Express$^+$ autoanalyzer (Chiron Diagnostics Co., Walpole, MA) using a commercially available, high-sensitivity CRP-Latex (II) X2 kit (Seiken Laboratories Ltd., Tokyo, Japan) that allowed detection of CRP concentrations as low as 0.001 mg/dL and as high as 32 mg/dL.

Fatty Acid Composition in Serum Phospholipids. Total lipids were extracted with chloroform:methanol (2:1, v/v) as described by Folch et al.[15] Phospholipids were methylated as described by Lepage and Roy after separation using thin-layer chromatography.[16] Fatty acid methyl esters were analyzed by gas chromatography (GC) (HP 6890A, Agilent Technologies, Inc., Santa Clara, CA) using an Omegawax 320 fused-silica capillary column (30 m × 0.32 mm with 0.25-μm film; Supelco Inc., Bellefonte, PA). Fatty acids were identified by comparing their retention times with those of standard fatty acid methyl esters. Percentages of individual fatty acids were calculated according to the peak areas relative to the total area (total fatty acid area was set at 100%).

Global (Nontargeted) Metabolic Profiling Analysis of Plasma by UPLC-Q-TOF MS. Sample Preparation and Analysis. Prior to analysis, 800 μL acetonitrile was added to 100 μL plasma. After shaking for 10 min at 4 °C, the mixture was centrifuged at 10 000 rpm for 5 min at 4 °C. The supernatant was freeze-dried at −70 °C and dissolved in 10% methanol. The supernatant was transferred into a vial, and a 7-μL sample was injected into the UPLC−Q-TOF MS (Waters, Milford, MA). The plasma extract was injected into an Acquity UPLC BEH C$_{18}$ column (2.1 × 50 mm, 1.7 μm; Waters) in line with the UPLC system and equilibrated with water containing 0.1% formic acid. Samples were eluted by an acetonitrile gradient containing 0.1% formic acid at a flow rate of 0.35 mL/min for 18 min, and metabolites were separated by UPLC, analyzed, and assigned by Q-TOF-MS (Waters). The Q-TOF was operated in ESI-positive mode.

The capillary and sampling cone voltages were set at 2.78 kV and 26 V, respectively. The desolvation flow was set to 700 L/h at a temperature of 300 °C, and the source temperature was set to 110 °C. The TOF-MS data were collected in the range of m/z 50−1000 with a scan time of 0.2 s and interscan delay

Table 1. Clinical Characteristics, Conventional Metabolites, and Inflammatory Markers in Normal-Weight and Overweight/Obese Men[a]

	normal-weight ($n = 30$)	overweight/obese ($n = 30$)	P value
Age (yrs)	9.5 ± 1.22	39.6 ± 1.24	0.994
Body mass index (kg/m^2)	20.9 ± 0.14	28.9 ± 0.20	<0.001
Body fat (%)	15.3 ± 0.52	27.7 ± 0.55	<0.001
Waist-hip ratio	0.86 ± 0.01	0.93 ± 0.01	<0.001
Cigarette smoker, *n* (%)	15 (53.6)	17 (58.6)	0.701
Alcohol drinker, *n* (%)	26 (92.9)	27 (93.1)	0.971
Systolic blood pressure (mm Hg)	115.1 ± 2.05	125.6 ± 2.00	0.001
Diastolic blood pressure (mm Hg)	66.2 ± 1.45	76.7 ± 1.65	<0.001
Glucose (mg/dl)[b]	91.4 ± 1.51	97.7 ± 1.85	0.031
Insulin (μU/ml)[b]	7.63 ± 0.48	13.7 ± 1.15	<0.001
[1]HOMA-IR[b]	1.72 ± 0.11	3.34 ± 0.30	<0.001
Free fatty acid (uEq/l)[b]	458.8 ± 48.8	618.2 ± 38.3	0.002
Triglyceride (mg/dl)[b]	85.5 ± 10.9	145.8 ± 11.1	<0.001
Total cholesterol (mg/dl)	183.6 ± 4.72	196.9 ± 5.84	0.047
LDL cholesterol (mg/dl)	114.2 ± 4.61	127.1 ± 5.02	0.032
HDL cholesterol (mg/dl)[b]	52.3 ± 2.22	40.7 ± 1.63	<0.001
Lp-PLA$_2$ activity (nmol/mL/min)	33.9 ± 2.69	34.0 ± 2.07	0.691
oxidized LDL (U/l)[b]	52.1 ± 4.01	63.9 ± 4.50	0.036
Adiponectin (μg/mL)[b]	7.35 ± 0.52	4.67 ± 0.29	<0.001
hs-CRP (mg/dl)[b]	0.85 ± 0.44	2.04 ± 0.63	<0.001
Malondialdehyde (nmol/mL) [b]	8.50 ± 0.30	9.26 ± 0.30	0.068
8-epi-PGF$_{2\alpha}$(pg/mg creatinine)[b]	1401.1 ± 127.9	1345.3 ± 71.0	0.643

[a] Data are mean ± S.E. [b] Log-transformed; tested by independent *t*-test with the Mann−Whitney U-test. [1]Insulin Resistance = {fasting insulin(μU/ml) × fasting glucose(mmol/l)}/22.5.

time of 0.02 s. The MS/MS spectra of metabolites were obtained by a collision energy ramp from 10−30 eV. The accurate mass and composition for the precursor and fragment ions were calculated and sequenced by MassLynx (Waters) incorporated in the instrument.

Data Processing and Identification of Metabolites. All information of MS data including retention times, m/z, and ion intensities was extracted by MarkerLynx software (waters, Milfold, MA) incorporated in the instrument, and the resulting MS data were assembled into a matrix. MarkerLynx parameters were set as follow: peak width at 5%, height 1s, intensity threshold 120 counts, mass window 0.04 amu, retention time window 0.15 min, noise elimination level 6 and mass tolerance 0.04 Da. Peak integration was performed using Apex Track integration. Metabolites were searched by the chemspider (www.chemspider.com) and human metabolome (www.hmdb.ca) databases or/and confirmed by standard samples based on both retention times and mass spectra.

Statistical Analysis. Statistical analyses were performed with SPSS ver12.0 (Statistical Package for the Social Sciences, SPSS Ins., Chicago, IL). The Kolmogorov−Smirnov test was used to determine the normality of the distribution, and skewed variables were logarithmically transformed for statistical analysis. For descriptive purposes, mean values are presented using untransformed values. Results are expressed as the mean ± SE. A two-tailed value of $P < 0.05$ was considered statistically significant. Pearson's correlation coefficients were used to examine the relationships between variables. Differences in clinical variables including mass intensities of plasma metabolites between the two groups were tested by independent *t*-test with the Mann−Whitney U-test. In addition, multivariate statistical analysis was performed using SIMCA-P$^+$ software version 12.0 (Umetrics, Umeå, Sweden). Cross-validation with seven cross-validation groups was used throughout to determine the number of principal components. Partial least-squares discriminant analysis (PLS-DA) was used as the classification method for modeling the discrimination between the overweight/obese and control subjects by visualizing the score plot or S-plot using the first and second PLS components. The goodness of the fit was quantified by R^2Y, while the predictive ability was indicated by Q^2Y. Generally, R^2Y—which describes how well the data in the training set are mathematically reproduced—varies between 0 and 1, where 1 indicates a model with a perfect fit. Moreover, models with Q^2Y greater than or equal to 0.5 are considered to have good predictive capability.[17]

Results

Clinical Characteristics and Conventional Metabolites and Markers. Clinical characteristics, conventional metabolites, and markers of normal-weight and overweight/obese men are shown in Table 1. No significant differences were observed in age, smoking, or alcohol consumption. As expected, overweight/obese men were heavier and had higher percentages of body fat and WHR than normal-weight men. Overweight/obese men had higher levels of glucose ($P = 0.031$), insulin ($P < 0.001$), HOMA-IR ($P < 0.001$), FFA ($P = 0.002$), triglyceride ($P < 0.001$), total cholesterol ($P = 0.047$), LDL-cholesterol ($P = 0.032$), ox-LDL ($P = 0.036$), and hs-CRP ($P < 0.001$) than lean men. Overweight/obese subjects had lower concentrations of serum HDL-cholesterol ($P < 0.001$) and plasma adiponectin ($P < 0.001$). However, no significant differences between groups were noted for Lp-PLA$_2$ activity, or for MDA or 8-epi-PGF$_{2\alpha}$ levels (Table 1).

Fatty Acid Composition of Serum Phospholipids Measured by GC. Compared to lean subjects, overweight/obese subjects showed higher proportions of stearic acid (18:0, $P = 0.035$), dihomo-γ-linolenic acid (20:3ω6, $P = 0.007$), and eicosatrienoic acid (20:3ω3, $P = 0.024$) in serum phospholipids (Table 2). Overweight/obese men showed a tendency toward

Table 2. Fatty Acid Composition in Serum Phospholipids[a]

	normal-weight ($n = 30$)	overweight/obese ($n = 30$)	P value
SFAs	54.5 ± 0.56	56.0 ± 0.53	0.051
14:0[b]	0.49 ± 0.03	0.51 ± 0.03	0.812
16:0	34.2 ± 0.33	34.6 ± 0.45	0.321
18:0[b]	17.1 ± 0.30	18.0 ± 0.30	0.035
MUFAs[b]	10.8 ± 0.31	10.6 ± 0.26	0.889
16:1[b]	0.52 ± 0.04	0.56 ± 0.02	0.068
18:1 (ω-9)	6.65 ± 0.23	6.53 ± 0.20	0.550
18:1 (ω-7)	1.77 ± 0.06	1.62 ± 0.06	0.044
PUFAs	25.0 ± 0.82	23.9 ± 0.58	0.182
Polyunsaturated ω-6	20.8 ± 0.66	19.9 ± 0.51	0.306
18:2 (ω-6)	13.8 ± 0.49	12.6 ± 0.42	0.084
18:3 (ω-6)[b]	0.21 ± 0.01	0.20 ± 0.01	0.838
20:2 (ω-6)[b]	0.38 ± 0.02	0.50 ± 0.18	0.082
20:3 (ω-6)	1.30 ± 0.07	1.60 ± 0.09	0.007
20:4 (ω-6)	4.89 ± 0.28	4.82 ± 0.26	0.787
Polyunsaturated ω-3[b]	4.21 ± 0.26	3.96 ± 0.23	0.446
18:3 (ω-3)[b]	0.10 ± 0.01	0.09 ± 0.01	0.290
20:3 (ω-3)[b]	0.05 ± 0.00	0.08 ± 0.03	0.024
Polyunsaturated ω-6/ω-3	5.34 ± 0.30	5.52 ± 0.36	0.712
δ−9 desaturase (18:1w9/18:0)	0.39 ± 0.02	0.37 ± 0.01	0.298
δ−9 desaturase (16:1w7/16:0)	0.02 ± 0.00	0.02 ± 0.00	0.106
δ−6 desaturase (18:3w6/18:2w6)	0.02 ± 0.00	0.02 ± 0.00	0.507
δ−5 desaturase (20:4ω6/20:3ω6)	3.87 ± 0.20	3.66 ± 0.65	0.009

[a] Data are mean ± S.E. [b] Log-transformed; tested by independent t-test with the Mann−Whitney U-test.

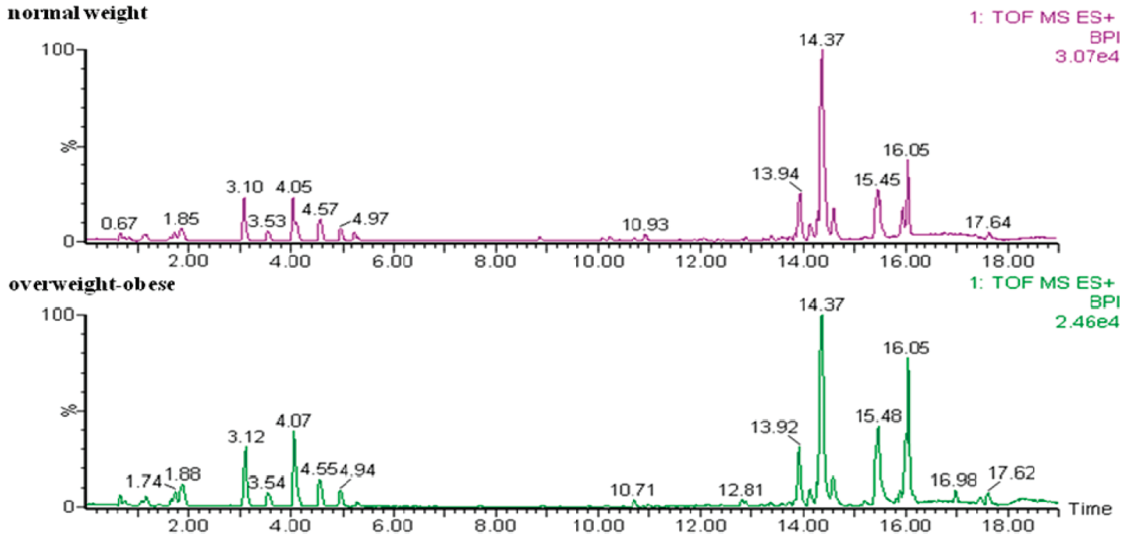

Figure 1. Raw BPI UPLC−MS data obtained from the plasma sample of normal-weight and overweight/obese men.

having higher levels of saturated fatty acids ($P = 0.051$), palmitolecic acid (16:1, $P = 0.068$), and eicosadienoic acid (20:2ω6, $P = 0.082$) and lower levels of linoleic acid (18:2ω6, $P = 0.084$) in serum phospholipids. However, overweight/obese men had lower levels of oleic acid (18:1ω7, $P = 0.044$) in serum phospholipids and lower δ−5 desaturase activity (20:4ω6/20:3ω6, $P = 0.009$).

Total Calorie and Nutrient Intake. Compared to normal-weight men, overweight/obese men had higher calorie intake (2385.3 ± 16.1, 2661.6 ± 21.2, $P < 0.001$), higher energy intake derived from fat (21.7 ± 0.20 vs 23.5 ± 0.18, $P < 0.001$), and higher saturated fat intake (10.2 ± 0.98 vs 16.2 ± 1.36, $P < 0.001$). However, overweight/obese individuals had lower ratios of polyunsaturated fatty acids (PUFAs) to saturated fatty acids (SFAs) (1.57 ± 0.13 vs 0.77 ± 0.08, $P < 0.001$) and lower energy intake derived from carbohydrates (61.7 ± 0.36 vs 59.4 ± 0.10, $P < 0.001$). There were no significant differences in energy intake derived from protein and the PUFA intake between groups.

Plasma Metabolic Profiling based on UPLC−Q-TOF MS. Nontargeted Metabolomics Pattern Analysis. Figure 1 shows the base peak intensity (BPI) chromatograms from the normal-weight and obese groups obtained from an analysis in positive-ion (ESI+) mode. To explain the maximum separation between defined class samples in the data set, the 347 variables obtained from normal-weight and overweight/obese men were performed with PLS-DA. The PLS-DA score plot (Figure 2a) showed a separation between overweight/obese and normal-weight men along the axes corresponding to first two PLS components. Our model describes 22.2% of the variation in X ($R^2X = 22.2\%$) and 81.7% of the variation in the response Y (class) ($R^2Y = 81.7\%$) and predicts 62.8% of the variation in the response Y

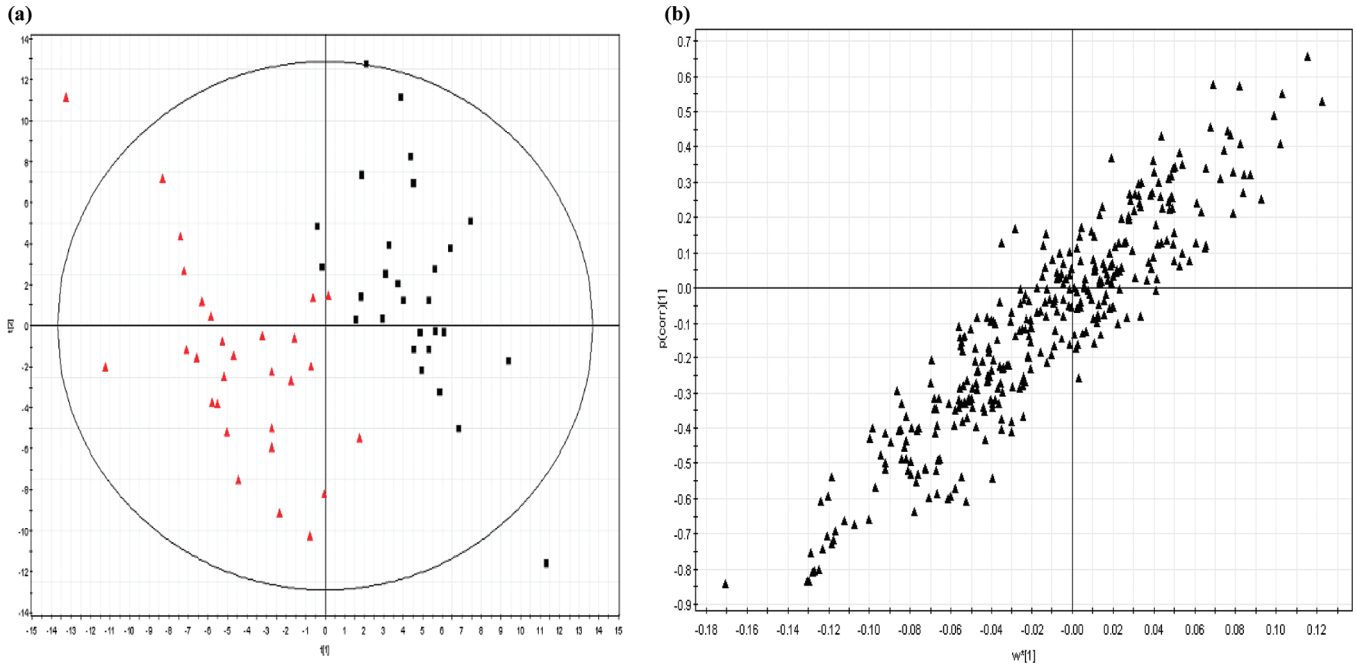

Figure 2. (a) Scores plots from PLS-DA models classifying overweight/obese (▲) and normal-weight men (■). (b) S-plot for weight (w*) and reliability correlation [p(corr)] from PLS-DA models.

Table 3. Identification and Quantification of Major Metabolites in Human Plasma Samples[a]

							normalized peak intensity (mean ± SE)		
no	retention time (min)	identity	formula $[M + H]^+$	actual mass	exact mass	mass error (ppm)	normal-weight	overweight/obese	*P*
1	0.7448	L-Carnitine	$C_7H_{15}NO_3$	162.1130	162.1131	−0.1	29.7 ± 2.03	24.1 ± 1.61	0.028
2	0.7553	Betaine	$C_5H_{11}NO_2$	118.0868	118.0869	−0.1	35.9 ± 1.39	32.1 ± 1.74	0.116
3	1.0744	L-Valine	$C_5H_{11}NO_2$	118.0868	118.0870	−0.2	27.1 ± 1.04	33.4 ± 1.34	0.001
4	1.1202	D-Pipecolic acid	$C_6H_{11}NO_2$	130.0868	130.0872	−0.4	19.0 ± 1.31	15.8 ± 0.93	0.122[b]
5	1.6495	L-Tyrosine	$C_9H_{11}NO_3$	182.0817	182.0811	0.6	44.2 ± 1.36	57.8 ± 2.13	<0.001
6	1.8355	L-Leucine	$C_6H_{13}NO_2$	132.1025	132.1017	0.8	104.5 ± 3.89	119.6 ± 4.09	0.003
7	2.3264	Propionyl carnitine	$C_{10}H_{19}NO_4$	218.1392	218.1392	0.0	10.9 ± 1.14	15.7 ± 0.75	<0.001
8	3.6766	Butyryl carnitine	$C_{11}H_{21}NO_4$	232.1549	232.1542	0.7	5.05 ± 0.69	9.02 ± 1.96	0.017
9	4.0552	L-tryptophan	$C_{11}H_{12}N_2O_2$	205.0977	205.0971	0.6	74.9 ± 2.35	83.0 ± 2.30	0.017
10	4.9525	unknown			349.1834		10.8 ± 0.29	15.3 ± 0.41	<0.001
11	6.3535	Hexanoyl carnitine	$C_{13}H_{25}NO_4$	260.1862	260.1858	0.4	5.04 ± 0.59	6.74 ± 0.37	0.046
12	8.8965	Octanoyl carnitine	$C_{15}H_{29}NO_4$	288.2175	288.2170	0.5	23.8 ± 1.87	27.1 ± 1.83	0.138
13	13.2888	Linoleyl carnitine	$C_{25}H_{45}NO_4$	424.3427	424.3413	1.4	26.8 ± 1.85	26.6 ± 1.51	0.962
14	13.3077	LysoPC (14:0)	$C_{22}H_{46}NO_7P$	468.3090	468.3073	1.7	31.7 ± 1.79	40.2 ± 2.98	0.046
15	13.3465	unknown			223.0654		7.33 ± 0.67	4.41 ± 0.55	<0.001
16	13.5665	Palmityl carnitine	$C_{23}H_{45}NO_4$	400.3427	400.3416	1.3	21.7 ± 1.64	23.3 ± 1.35	0.987
17	13.9182	LysoPC (18:2)	$C_{26}H_{50}NO_7P$	520.3403	520.3398	0.5	533.3 ± 28.5	525.5 ± 28.9	0.702
18	14.5952	LysoPC (18:1)	$C_{26}H_{52}NO_7P$	522.3560	522.3550	1.0	380.0 ± 19.3	284.5 ± 15.6	0.001
19	14.8311	LysoPC (20:2)	$C_{28}H_{54}NO_7P$	548.3716	548.3716	0.0	6.53 ± 0.31	6.23 ± 0.42	0.786
20	15.2295	unknown			350.2675		10.1 ± 0.62	6.47 ± 0.77	0.005
21	15.4533	LysoPC (18:0)	$C_{26}H_{54}NO_7P$	524.3716	524.3713	0.3	529.8 ± 15.5	595.2 ± 23.7	0.015
22	15.9274	unknown			338.3417		244.6 ± 33.8	75.7 ± 29.0	<0.001

[a] Data are mean ± S.E. [b] Log-transformed; tested by independent *t*-test with the Mann-Whitney U-test.

($Q^2Y = 62.8\%$) for two-component model. The permutation test with a permutation number of 200 was performed and indicated a R^2 intercept value of 0.613 and a Q^2 intercept value of −0.0399.

The score plot indicated that the overweight/obese group was clearly separated from the normal-weight group, and the S-plot (Figure 2b) indicated that each metabolite contributed to separation between the normal-weight and overweight/obese groups along the axes corresponding to the combined weight (w*) and reliability correlation [p(corr)].

Identification of Possible Biomarkers. To identify possible plasma biomarkers, the S-plot of PLS-DA, which complements the score plot of PLS-DA by the transposed matrix calculation, was used. Table 3 shows 18 metabolites. Among the total 18 metabolites measured in the plasma, seven metabolites [betaine, D-pipecolic acid, octanoyl carnitine, linoleyl carnitine, palmityl carnitine, lyso-phosphatidylcholine (lysoPC) (18:2), lysoPC (20:2)] were not significantly different between normal-weight and overweight/obese men. The normalized peak intensity of 9 metabolites, such as L-valine ($P = 0.001$),

L-tyrosine ($P < 0.001$), L-leucine ($P = 0.003$), propionyl carnitine ($P < 0.001$), butyryl carnitine ($P = 0.017$), L-tryptophan ($P = 0.017$), hexanoyl carnitine ($P = 0.046$), lysoPC (C14:0) ($P = 0.046$), and lysoPC (C18:0) ($P = 0.015$) were significantly higher in the overweight/obese group than in the normal-weight group, whereas the values for L-carnitine ($P = 0.028$) and lysoPC (C18:1) ($P < 0.001$) were lower in the overweight/obese group.

Discussion

This metabolomics study of overweight/obese men based on UPLC/Q-TOF MS identified three potential plasma markers: lysoPC (C14:0), lysoPC (C16:0), and lysoPC (C18:1), and confirmed eight known metabolites, that is, two BCAA (valine and leucine), two essential amino acids (phenylalanine and tryptophan), and key compounds of fatty acid synthesis and oxidation (carnitine, propionyl-, butyryl-, and hexanoyl-carnitine). LysoPC has different species based on fatty acid chain length and degree of saturation, which could have different physical and biological properties.[18] This important issue is largely unexploited in humans. In this study, among the five lysoPC species analyzed, the levels of lysoPC (C18:2) and lysoPC (C20:2) were not significantly different in overweight/obese versus lean men. Plasma levels of lysoPC (C14:0) and lysoPC (C18:0) were significantly higher, whereas lysoPC (C18:1) levels were lower in overweight/obese than lean subjects. In the Zucker (fa/fa) obese rat strain, lysoPC (C16:0), lysoPC (C18:1), 1-octadecyl-sn-glycerophosphocholine, and lysoPC (C18:0) were found in higher concentrations compared with normal wild-type rats.[19] Galili et al.[20] also found that plasma lysoPC (C18:0) levels were higher in pigs fed a high-fat/high-calorie diet (4.31 kcal/g, 20% lard) compared with pigs fed normal chow (0.81 kcal/g), with no difference in the levels of lysoPC (C16:0).

LysoPC constitutes only 1−5% of the total PC content of non-oxLDL; however, as much as 40−50% of the PC contained within the LDL molecule is converted to lysoPC during LDL oxidation via two different pathways.[21] Circulating lysoPC is generated predominantly by the activity of lecithin-cholesterol acytransferase (LCAT), which transfers a fatty acid from PC to cholesterol.[22,23] Moreover, PLA_2, including secretory PLA_2 (sP-LA_2)[24] and Lp-PLA_2,[25−27] hydrolyzes PC, simultaneously generating one molecule of lysoPC and one molecule of arachidonic acid.[18,28] Therefore, lysoPC accumulation reflects increased production via PLA_2-catalyzed PC hydrolysis. Since Lp-PLA_2 activity and urinary excretion of 8-epi-$PGF_{2\alpha}$ showed a positive correlation ($r = 0.325$, $P = 0.021$) and these two variables were not different in overweight/obese versus lean men, significant differences in three lysoPC species (C14:0, C18:0, and C18:1) might be partly due to the altered level and/or composition of constituent acyl groups of lysoPC during oxidative modification rather than increased production from PLA_2-catalyzed PC hydrolysis. This hypothesis is consistent with a previous report that the level of Lp-PLA_2 is independent of traditional risk factors, BMI and insulin resistance.[29] Chen et al.[30] found that the high levels of LDL found in the plasma of hyperlipidemic patients, coupled with its enhanced ability to generate long chain species of lysoPC during oxidative modification, are important factors for the development of atherosclerosis in hyperlipidemic patients. In this study, overweight/obese men showed 22% higher ox-LDL concentrations with weakly but significantly higher total cholesterol (7%) and LDL-cholesterol (11%) levels than lean men.

Similar to significant differences in the levels of lysoPC (C18:0) between the two groups, overweight/obese men also showed higher levels of stearic acid (18:0) in serum phospholipids. Furthermore, they showed a tendency toward higher levels of total SFAs and palmitoleic acid (16:1ω7) and a trend toward lower linoleic acid (18:2ω6) levels. Fatty acid composition in serum phospholipids was found to be a biochemical marker of long-term fatty acid intake, especially for essential fatty acids including linoleic acid (18:2ω6).[31,32] Palmitoleic acid (16:1ω7) is almost exclusively derived from palmitic acid (16:0) by desaturation of $\delta-9$ desaturase ω7,[33] and 16:1ω7 by long-chain elongase is extended to 18:1ω7, a putative marker of carbohydrate intake.[34−36] Higher saturated fat intake with a lower ratio of PUFAs to SFAs and relatively lower carbohydrate levels in overweight/obese compared with lean subjects in this study could also partly explain higher levels of lysoPC (C18:0) and stearic acid (18:0) and lower levels of oleic acid (18:1ω7) in serum phospholipids.

Serum fatty acid composition may modulate insulin action, and increased serum SFA concentrations are known to impair glucose metabolism, potentially causing diabetes.[37] Not surprisingly, we found the overweight/obese men to be more insulin resistant than the lean men, and we also observed that the levels of the branched-chain amino acids (BCAAs) valine and leucine were 23% and 14% higher, respectively, in overweigh/obese men compared to lean men. This result is consistent with the recent identification of a cluster of obesity-associated changes in specific amino acids and short-chain acylcarnitine in obese compared to lean subjects; these changes are associated with insulin resistance.[5] Higher levels of aromatic amino acids, tyrosine and tryptophan, in overweight/obese men compared to lean men support the previous suggestion that both BCAAs and aromatic amino acids compete for transport into cells by the large neutral amino acid transporter.[38] Furthermore, findings of higher levels of propionyl-, butyryl-, and hexanoyl-carnitines in obese men are in agreement with the report of Newgard et al.[32] that byproducts or intermediates of BCAA catabolism increase specific C3 and C5 acylcarnitine levels. In addition, BCAAs are known to potentiate glucose-stimulated insulin secretion and results in the exaggerated insulin secretion response to glucose in obese subjects.[5] A number of the obesity-related changes in traditional biomarkers described in this study confirm prior studies, including higher levels of insulin, glucose, and HOMA-IR and lower levels of adiponectin; increases in inflammatory/cardiovascular risk markers, such as CRP;[39−41] and abnormal linoleic acid metabolism that include higher levels of dihomo-γ-linolenic acid (20:3ω6) and higher δ-6 desaturase activity.[42]

The fatty acid composition of serum phospholipids has been shown to mirror dietary fatty acid patterns.[31] In experimental animals, chronic feeding of saturated fat is more deleterious to insulin sensitivity than either monounsaturated fatty acids (MUFAs) or PUFAs.[43] Epidemiological studies also support a positive association between saturated fat intake and risk of insulin resistance, diabetes,[37] and coronary artery disease.[44] Even though the subjects in this study had lower fat energy intake, high saturated fat intake and higher lysoPC (C14:0 and C18:0) levels in overweight and obese men suggest that the amount of saturated fat in the diet influences SFA levels in serum phospholipids as well as the degree of saturation of the constituent acyl group of plasma lysoPC. In recent decades, Koreans have increased the percentage of calories from fat in their diets from 7.2% in 1969 to 18.5% in 2007,[45] and the

mortality rate of ischemic heart disease has rapidly increased from 6.8% in 1988 to 29.5% in 2007; diabetes has similarly increased from 7.4% in 1988 to 22.9% in 2007.[46] Therefore, substitution of unsaturated for saturated fat in the diet may improve insulin sensitivity as well as the physical and biological properties of lysoPC, ultimately leading to reduce diabetic and cardiovascular risk.

Our results have the limitation of all cross-sectional and observational studies. We evaluated associations rather than prospective predictions; thus, the causal relationship of the identified biomarkers and the exact internal mechanisms of the changes of the metabolites in obesity are still unclear. In addition, a large number of markers were detected by UPLC−MS but most remain unidentified at present. Unlike GC−MS for which large databases exist, the use of LC−MS-based techniques for metabolomics research is still in its infancy, and the databases of endogenous biomolecules have not yet been constructed.[6]

Despite these limitations, the current study showed a cluster of obesity-associated changes in metabolites, including three lysoPC species (C14:0, C18:0, and C18:1), specific amino acids (two BCAA, two aromatic amino acids), L-carnitine, and acylcarnitines (propionyl-, butyryl-, and hexanoyl-carnitine) using a UPLC−Q-TOF MS-based metabolomics strategy and multivariate data analysis. The differences in these metabolic profiles between the lean and overweight/obese may provide a better understanding of the metabolic changes of obesity, which may be important for future clinical diagnosis and treatment.

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