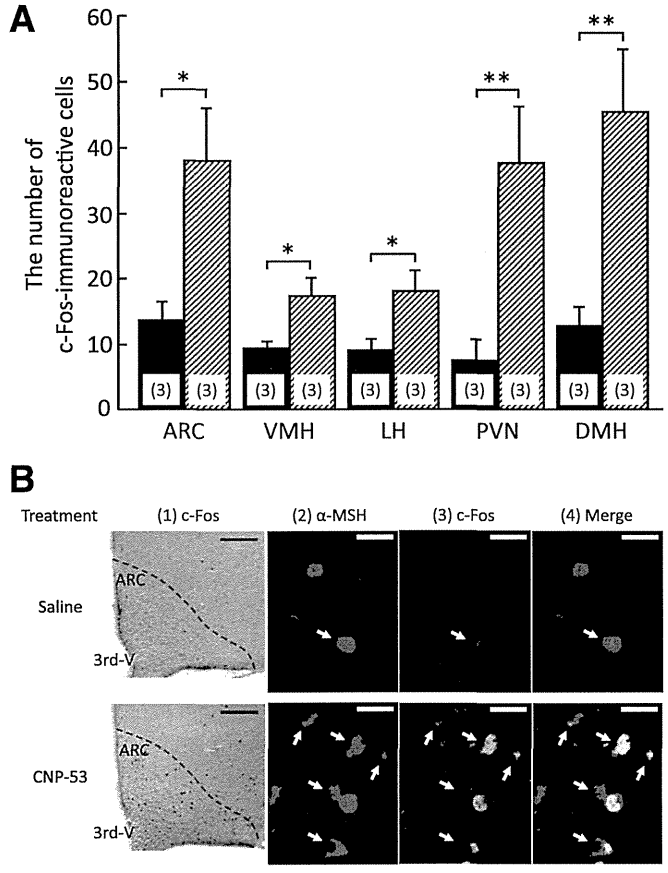

FIG. 4. The c-Fos–immunoreactive cells in the hypothalamus after intracerebroventricular administration of CNP-53 (1.5 nmol/mouse). *A*: Number of c-Fos–immunoreactive cells after saline and CNP-53 treatments. Data represent mean ± SEM. The number of mice is given in parentheses. Significant differences: *$P < 0.05$, **$P < 0.01$. *B*: c-Fos–immunoreactive cells induced by intracerebroventricular administration of saline and CNP-53 (1). 3rd-V, the third ventricular. Scale bars, 100 μm. Coexistence of α-MSH (red) and c-Fos (green) immunoreactivity in the ARC (2–4) after saline (*upper*) and CNP-53 (1.5 nmol/mouse; *lower*) treatments. White arrows indicate cells expressing both α-MSH and c-Fos immunoreactivity. 3rd-V, the third ventricular. Scale bars, 20 μm.

discrepancy may lie in the experimental condition, time course, and regional specificity. To clarify this discrepancy, further examinations will be required.

This study demonstrated that the intracerebroventricular administration of CNP significantly suppressed the nocturnal food intake. Robust feeding during the nocturnal phase of the daily light–dark cycle was demonstrated to be attributed to the upregulation of NPY and its receptors (13). These findings indicate that CNP may decrease food intake in the nocturnal phase via suppression of NPY action.

In the current study, CNP significantly suppressed the increase in food intake induced by ghrelin, an orexigenic hormone secreted by the stomach (14). NPR-B, a CNP receptor, has been identified in appetite-regulating regions, such as the ARC, VMH, PVN, DMH, and LH (15). The systemic administration of ghrelin significantly increased NPY and AgRP expression in the ARC of the hypothalamus in fed and fasted rats (15), resulting in hyperphagia. The intracerebroventricular injection of melanotan II caused a significant decrease in ghrelin-induced food intake (16). These findings suggest that the actions of ghrelin are modulated by α-MSH and NPY systems. Furthermore, plasma ghrelin and hypothalamic ghrelin receptor mRNA expression are reported to be increased after fasting (17,18). These findings suggest the possibility that intracerebroventricular administration of CNP activates the melanocortin system, which subsequently inhibits the action of NPY, resulting in a reduced increase of food intake induced by ghrelin.

To assess which hypothalamic nucleus is involved in the anorexigenic action of CNP, a marker for neuronal activity, c-Fos expression in the hypothalamus was examined after intracerebroventricular administration of CNP-53. The intracerebroventricular administration of CNP-53 significantly increased the number of c-Fos–expressing cells in several hypothalamic nuclei, such as ARC, PVN, DMH, VMH, and LH, indicating that CNP-53 directly or indirectly stimulates neurons in these hypothalamic nuclei. Especially in the ARC, the result was an increased number of c-Fos–immunoreactive cells containing α-MSH immunoreactivity, indicating that CNP stimulates α-MSH–containing neurons. This possibility is supported by the finding that the suppressive action of CNP-53 on food intake was blocked by concomitant administration of SHU9119, an MC3R/MC4R antagonist.

The current study has demonstrated the anorexigenic action of intracerebroventricular administration of CNP via activation of the melanocortin system. To define the precise effect of CNP in the brain on food intake, further investigation using mice with inducible brain-specific deletion of CNP or NPR-B/NPR-C will be required.

From the present findings, we postulate the possible mechanism for anorexigenic action of exogenous CNP to be as follows: CNP directly or indirectly acts on α-MSH–containing neurons and subsequently stimulates α-MSH release, resulting in suppression of food intake induced by NPY and ghrelin. This possible mechanism may apply to the suppressive effects of CNP on food intake after fasting and in the nocturnal phase. Further work is needed to define the pathophysiological significance of brain CNP in regulation of food intake.

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N.Y.-G. and G.K. performed experiments, contributed to discussion, and wrote the manuscript. K.E., M.I., Y.O., Y.Y., T.K., A.Y., N.S.-A., H.A., and K.H. contributed to discussion. K.N. contributed to discussion, and reviewed and edited the manuscript. K.N. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Original Article

Replication Study of 15 Recently Published Loci for Body Fat Distribution in the Japanese Population

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Aim: Visceral fat accumulation plays an integral role in morbidity and mortality rates by increasing the risk of developing metabolic disorders such as type 2 diabetes, dyslipidemia, and hypertension. New genetic loci associated with fat distribution, measured by waist-hip ratios and computed tomography (CT), have recently been identified by genome-wide association studies in European-descent populations. This study used CT to investigate whether single nucleotide polymorphisms (SNPs) that confer susceptibility to fat distribution are associated with visceral fat area (VFA) and subcutaneous fat area (SFA) in the Japanese population.

Methods: We measured the VFAs and SFAs of 1424 obese Japanese subjects (BMI ≥ 25 kg/m^2, 635 men and 789 women) that were genotyped at 15 SNPs, namely, *TBX15* rs984222, *DNM3* rs1011731, *LYPLAL1* rs4846567, *GRB14* rs10195252, *NISCH* rs6784615, *ADAMTS9* rs6795735, *CPEB4* rs6861681, *LY86* rs1294421, *VEGFA* rs6905288, *RSPO3* rs9491696, *NFE2L3* rs1055144, *ITPR2* rs718314, *HOXC13* rs1443512, *ZNRF3* rs4823006 and *THNSL2* rs1659258.

Results: The G-allele of *LYPLAL1* rs4846567 was borderline associated with an increased ratio of VFA to SFA (V/S ratio; $p=0.0020$). *LYPLAL1* rs4846567 had a stronger effect on the V/S ratio in women ($p=0.0078$) than in men ($p=0.12$); however, neither result was significant after Bonferroni correction for multiple comparisons. *NISCH* rs6784615 was nominally associated with increased VFA ($p=0.040$) and V/S ratio ($p=0.020$). The other SNPs analyzed were not significantly associated with body mass index (BMI), VFA, or SFA.
Conclusion: Our results suggest that *LYPLAL1* rs4846567 and *NISCH* rs6784615 may influence fat distribution in the Japanese population.

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Key words; *LYPLAL1*, Visceral fat area, Subcutaneous fat area, Computed tomography, Japanese subjects

Introduction

Metabolic syndrome is a combination of medical disorders, including central obesity, impaired glucose tolerance, dyslipidemia, and hypertension, that increase the risk of cardiovascular disease morbidity and mortality[1]. Several studies have indicated that intra-abdominal adipose tissue plays a central role in metabolic syndrome; accumulated visceral adipose tissue may lead to alterations in the plasma levels of adipocytokines, thereby resulting in the development of dyslipidemia, hypertension, and insulin resistance[2, 3]. Intra-abdominal fat accumulation (central adiposity) is determined by waist circumference, waist-hip ratio, biological impedance, or the visceral fat area (VFA) measured using computed tomography (CT)[1, 4, 5]. Waist circumference and the waist-hip ratio are commonly used because they are simple and convenient to measure; however, VFA measured using CT is a far more precise method of assessing fat distribution[1, 4, 5].

There is an abundance of evidence showing that body fat distribution is influenced by genetic loci[6-9]. Genome-wide association studies (GWAS) have been conducted to identify the loci linked to waist circumference and the waist-hip ratio in Caucasian populations[10-12]. In a previous study, we examined the associations of 6 reported waist circumference or waist-hip ratio loci[10, 11] with VFA, and determined that the rs1558902 and rs1421085 genotypes of the fat mass- and obesity-associated gene (*FTO*) were significantly associated with subcutaneous fat area (SFA)[13]. Very recently, rs1659258 near the threonine synthase-like 2 (*THNSL2*) gene was identified to be associated with VFA in women by GWAS for VFA and SFA using CT[14].

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Aim

In this study, we investigated whether the 14 recently reported novel waist-hip ratio-associated SNPs[12] and VFA-associated rs1659258[14] also affect VFA or the ratio of VFA to SFA (V/S ratio) in the Japanese population, which is an important factor in the development of metabolic syndrome.

Methods

Study Subjects

We enrolled 1424 Japanese subjects both with and without metabolic abnormalities such as hypertension, dyslipidemia, and type 2 diabetes, who had visited outpatient clinics to undergo treatment for obesity (≥25 kg/m^2). Obesity was diagnosed according to Japanese obesity criteria[15]. The patients agreed to undergo CT testing (in the supine position) to determine VFA and SFA values at the umbilical level (L4-L5), as previously reported[16]. The VFA and SFA values, and the V/S ratio were calculated using the FatScan software program (N2system, Osaka, Japan)[16]. Clinical data were recorded at the first visit to the hospital; the clinical characteristics of the subjects are summarized in **Table 1**. Written informed consent was obtained from each subject, and the protocol was approved by both the ethics committee of each participating institution and of Kyoto University.

DNA Extraction and SNP Genotyping

Genomic DNA was extracted from the blood samples collected from each subject using a Genomix kit (Talent Srl, Trieste, Italy). We selected 15 SNPs

that were recently identified as loci associated with waist-hip ratios[12] and with VFA[14] by GWAS meta-analysis in European-descent populations, and constructed Invader probes (Third Wave Technologies, Madison, WI, USA) for each. The 15 selected SNPs were rs984222 in the T-box 15 (*TBX15*) gene, rs1011731 in the dynamin 3 (*DNM3*) gene, rs4846567 in the lysophospholipase-like 1 (*LYPLAL1*) gene, rs10195252 in the growth factor receptor-bound protein 14 (*GRB14*) gene, rs6784615 in the nischarin (*NISCH*) gene, rs6795735 in the ADAM metallopeptidase with thrombospondin type 1 motif, 9 (*ADAMTS9*) gene, rs6861681 in the cytoplasmic polyadenylation element binding protein 4 (*CPEB4*) gene, rs1294421 in the lymphocyte antigen 86 (*LY86*) gene, rs6905288 in the vascular endothelial growth factor A (*VEGFA*) gene, rs9491696 in the R-spondin 3 (*RSPO3*) gene, rs1055144 in the nuclear factor (erythroid-derived 2)-like 3 (*NFE2L3*) gene, rs718314 in the inositol 1,4,5-trisphosphate receptor, type 2 (*ITPR2*) gene, rs1443512 in the homeobox C13 (*HOXC13*) gene, rs4823006 in the zinc and ring finger 3 (*ZNRF3*) gene, and rs1659258 in the *THNSL2* gene. The SNPs were genotyped using Invader assays as previously described[17]. The success rate of these assays was >99.5%.

Statistical Analysis

For the additive model, we categorized the genotypes as 0, 1, or 2 depending on the number of copies of the previously alluded to waist-hip ratio-associated and VFA-associated[12, 14] risk alleles present. Multiple linear regression analyses were performed to test the independent effect per allele of each SNP on BMI, VFA, SFA, and the V/S ratio by accounting for the effects of the other variables (i.e., age and gender). The BMI, VFA, SFA, and V/S ratio values were logarithmically transformed before performing multiple linear regression analysis. Hardy-Weinberg equilibrium was assessed using the χ^2-test[18]. Statistical analyses were performed using R software (http://www.r-project.org/). *P* values were assessed after Bonferroni correction for multiple comparisons, and $p<0.0011$ [0.05/15 (total SNP number)/3 (number of traits)] was considered significant.

Results

The clinical characteristics and genotypes of the subjects are shown in **Table 1** and **2**, respectively. All the SNPs were in Hardy-Weinberg equilibrium, except rs984222 ($p=0.045$), and the minor allele frequencies did not diverge from those reported in the HapMap database. The BMI, VFA, SFA, and V/S ratio values for each SNP genotype are shown in **Table 3**. Multiple linear regression analyses of the anthropometric parameters with respect to the 15 SNPs analyzed are shown in **Table 4**. No SNP was significantly associated with BMI, VFA, SFA, or V/S ratio. The G-allele of rs4846567 in the *LYPLAL1* gene was borderline associated with the V/S ratio ($p=0.0020$). *NISCH* rs6784615 was nominally associated with increased VFA and the V/S ratio ($p<0.05$). We did not detect a significant association between 15 SNPs and fat distribution, which may have been due to the limitation of the sample power; therefore, we conducted power analysis of linear regression (additive model) with the significance level set at $p<0.05$, using age and gender as explanatory parameters. The estimated effect sizes per allele (regression coefficients) for the logarithmically transformed VFA, SFA, and V/S ratio values were 0.017, 0.010, and 0.028, respectively, based on the rs4846567 values (**Table 3**). The power of our statistical test was calculated on the basis of these estimated effect sizes and by performing 10,000 simulations. When the allele frequency was assumed to be 0.20, the power was estimated to be 0.30 for VFA, 0.19 for SFA, and 0.59 for the V/S ratio; however, when the allele frequency was assumed to be 0.10, the respective powers were estimated to be 0.18, 0.12, and 0.38.

BMI, VFA, SFA, and the V/S ratio are known to be affected by gender; thus, we compared the rs4846567 alleles with the fat distribution parameters (BMI, VFA, SFA, and V/S ratio) in men and women independently (**Table 5**). Association between *LYPLAL1* rs4846567 and the V/S ratio was not significant in men ($p=0.12$) and was above the predetermined significance threshold in women ($p=0.0078$). The effect of other SNPs on fat distribution was also analyzed separately in men and women (**Supplementary Table 1** and **2**). The G allele of rs9491696 in the *RSPO3* gene were nominally associated with SFA in women ($p=0.038$).

Multiple linear regression analysis was performed to explore the effect of various confounding factors and clinical parameters, as well as rs4846567 genotype, on the V/S ratio. The V/S ratio was significantly correlated with age, fasting plasma glucose, insulin, homeostasis model assessment-insulin resistance index (HOMA-IR), triglycerides, high-density lipoprotein (HDL)-cholesterol, systolic blood pressure, and diastolic blood pressure (**Supplementary Table 3**). BMI and total cholesterol were not significantly correlated with the V/S ratio. Based on the results of Spearman's rank correlation coefficient calculations, we used the V/S ratio as the dependent variable, and included

Table 1. Clinical characteristics of the subjects

	Men	Women	Total
n	635	789	1424
Age (years)	48.6 ± 12.5	52.3 ± 11.3	50.7 ± 12.0
BMI (kg/m^2)	29.9 ± 6.0	28.2 ± 5.2	29.0 ± 5.6
VFA (cm^2)	153.9 ± 66.6	102.6 ± 54.1	125.5 ± 65.1
SFA (cm^2)	205.5 ± 108.3	243.3 ± 97.7	226.5 ± 104.3
Fasting plasma glucose (mg/dL)	109.4 ± 31.9	108.4 ± 36.5	108.9 ± 34.5
Fasting insulin (μU/mL) (n=1312)	13.6 ± 17.9	10.4 ± 10.3	11.8 ± 14.3
HOMA-IR (n=1312)	3.9 ± 7.5	2.9 ± 3.8	3.4 ± 5.8
Total cholesterol (mg/dL)	211.4 ± 37.1	220.1 ± 38.6	216.2 ± 38.2
Triglycerides (mg/dL)	170.0 ± 147.9	120.6 ± 80.8	142.6 ± 118.1
HDL-cholesterol(mg/dL)	51.7 ± 13.9	61.0 ± 16.0	56.8 ± 15.8
Systolic blood pressure (mmHg)	131.4 ± 16.8	130.5 ± 18.4	130.9 ± 17.8
Diastolic blood pressure (mmHg)	84.7 ± 12.5	80.7 ± 11.2	82.4 ± 12.0

Abbreviations: BMI, body mass index; HOMA-IR; homeostasis model assessment-insulin resistance index; SFA, subcutaneous fat area; HDL, high density lipoprotein; VFA, visceral fat area.
HOMA-IR was assessed as fasting insulin (μU/mL × fasting plasma glucose/405. Data are represented as the mean ± s.d.

Table 2. Genotypic characteristics of the subjects

SNP ID	Chr	Position (Build 36.3)	Nearby gene	Alleles 1/2	Risk allele	Genotype	Risk allele frequency	HWE p-value
rs984222	1	119,305,366	*TBX15*	G/C	G	232/639/550	0.39	0.045
rs1011731	1	170,613,171	*DNM3*	A/G	G	1151/257/14	0.10	0.93
rs4846567	1	217,817,340	*LYPLAL1*	G/T	G	531/657/231	0.61	0.25
rs1659258	2	88,440,703	*THNSL2*	A/G	A	1036/349/37	0.85	0.25
rs10195252	2	165,221,337	*GRB14*	C/T	T	11/203/1207	0.92	0.45
rs6784615	3	52,481,466	*NISCH*	C/T	T	0/47/1377	0.98	0.53
rs6795735	3	64,680,405	*ADAMTS9*	C/T	C	35/370/1017	0.15	0.85
rs6861681	5	173,295,064	*CPEB4*	A/G	A	12/260/1151	0.10	0.52
rs1294421	6	6,688,148	*LY86*	G/T	G	36/387/996	0.16	0.83
rs6905288	6	43,866,851	*VEGFA*	A/G	A	867/495/58	0.78	0.22
rs9491696	6	127,494,332	*RSPO3*	G/C	G	356/694/369	0.50	0.41
rs1055144	7	25,837,634	*NFE2L3*	C/T	T	302/726/393	0.53	0.33
rs718314	12	26,344,550	*ITPR2*	A/G	G	94/548/781	0.74	0.87
rs1443512	12	52,628,951	*HOXC13*	A/C	A	47/421/951	0.18	0.96
rs4823006	22	27,781,671	*ZNRF3*	A/G	A	170/610/644	0.33	0.17

Abbreviations: Chr, chromosome; HWE, Hardy-Weinberg equilibrium; SNP, single-nucleotide polymorphism.

rs4846567 genotype, age, gender, fasting plasma glucose, insulin, HOMA-IR, triglycerides, HDL-cholesterol, systolic blood pressure, and diastolic blood pressure as explanatory variables in the original model of multiple linear regression. Stepwise multiple linear regression analysis (both forward selection and backward elimination) revealed that rs4846567 genotype (p=0.00029), age ($p<2\times10^{-16}$), gender ($p<2\times10^{-16}$), fasting plasma glucose ($p=2.8\times10^{-5}$), triglycerides ($p=8.7\times10^{-14}$), and HDL-cholesterol (p=0.00025) were significantly associated with the V/S ratio (**Supplementary Table 4**).

Numerous waist-hip ratio-susceptible SNPs are evidently associated with metabolic traits[12], and we examined the effects of 15 such SNPs on various metabolic traits. The risk allele (G-allele) rs4846567 in the *LYPLAL1* gene was nominally associated with decreased fasting plasma glucose ($p<0.05$), and rs6925288 in the *VEGFA* gene was associated with increased diastolic blood pressure ($p<0.05$; **Supple-**

Table 3. Mean BMI, VFA, SFA, and V/S ratio of the 15 fat distribution risk variants

	Mean ± s.d.					
	BMI (kg/m²)			VFA (cm²)		
	Genotype			Genotype		
SNP ID	11	12	22	11	12	22
rs984222	29.3 ± 6.0	28.9 ± 5.5	28.9 ± 5.7	124.7 ± 63.0	126.3 ± 66.6	124.9 ± 64.6
rs1011731	29.0 ± 5.7	28.7 ± 5.2	28.0 ± 4.1	125.4 ± 66.0	126.4 ± 62.3	116.3 ± 50.3
rs4846567	28.6 ± 5.0	29.2 ± 5.9	29.0 ± 6.2	127.9 ± 64.4	126.0 ± 67.7	118.0 ± 59.3
rs1659258	28.9 ± 5.8	29.0 ± 5.2	30.4 ± 6.2	124.1 ± 64.8	129.6 ± 65.9	127.1 ± 66.5
rs10195252	28.5 ± 4.9	29.0 ± 5.8	29.0 ± 5.6	168.2 ± 86.8	121.0 ± 58.1	125.8 ± 66.0
rs6784615	-	27.8 ± 4.6	29.0 ± 5.7	-	119.2 ± 62.2	125.7 ± 65.2
rs6795735	27.1 ± 5.0	28.9 ± 5.4	29.1 ± 5.7	101.4 ± 52.9	129.2 ± 67.4	125.0 ± 64.6
rs6861681	28.6 ± 5.4	28.4 ± 5.9	29.1 ± 5.6	121.0 ± 74.3	126.9 ± 71.4	125.3 ± 63.6
rs1294421	28.3 ± 4.5	29.7 ± 6.7	28.7 ± 5.2	117.5 ± 66.3	124.1 ± 67.0	126.5 ± 64.5
rs6905288	29.0 ± 5.8	28.9 ± 5.3	29.8 ± 5.3	125.5 ± 66.1	125.2 ± 63.7	128.2 ± 65.3
rs9491696	29.2 ± 5.9	28.9 ± 5.6	29.0 ± 5.5	125.6 ± 66.0	124.2 ± 63.0	128.0 ± 68.5
rs1055144	29.2 ± 6.2	28.8 ± 5.3	29.1 ± 5.8	123.5 ± 68.8	125.4 ± 63.7	127.4 ± 65.2
rs718314	28.2 ± 5.2	28.8 ± 5.2	29.2 ± 6.0	123.1 ± 58.2	124.2 ± 64.8	126.7 ± 66.3
rs1443512	29.6 ± 6.9	28.7 ± 4.9	29.0 ± 5.9	121.3 ± 63.4	124.2 ± 64.3	126.3 ± 65.7
rs4823006	28.2 ± 5.0	29.1 ± 5.7	29.1 ± 5.7	115.8 ± 60.8	132.1 ± 68.0	121.8 ± 62.9

	Mean ± s.d.					
	SFA (cm²)			V/S		
	Genotype			Genotype		
SNP ID	11	12	22	11	12	22
rs984222	233.4 ± 103.5	224.3 ± 105.0	225.9 ± 104.0	0.61 ± 0.39	0.64 ± 0.41	0.62 ± 0.36
rs1011731	228.2 ± 106.3	220.3 ± 95.9	196.9 ± 76.8	0.62 ± 0.39	0.65 ± 0.39	0.65 ± 0.33
rs4846567	219.5 ± 101.0	230.2 ± 104.5	231.6 ± 110.6	0.67 ± 0.41	0.61 ± 0.37	0.59 ± 0.38
rs1659258	225.5 ± 103.6	228.1 ± 105.5	238.1 ± 114.1	0.63 ± 0.39	0.65 ± 0.37	0.59 ± 0.34
rs10195252	187.8 ± 56.5	220.9 ± 100.0	227.7 ± 105.3	0.95 ± 0.52	0.62 ± 0.36	0.63 ± 0.39
rs6784615	-	209.6 ± 91.7	227.0 ± 104.6	-	0.61 ± 0.32	0.63 ± 0.39
rs6795735	208.2 ± 118.2	230.9 ± 103.7	225.3 ± 104.0	0.58 ± 0.36	0.63 ± 0.40	0.63 ± 0.38
rs6861681	255.6 ± 104.9	214.2 ± 99.1	229.1 ± 105.2	0.47 ± 0.21	0.68 ± 0.46	0.62 ± 0.37
rs1294421	219.3 ± 87.4	239.3 ± 115.1	221.7 ± 99.6	0.58 ± 0.31	0.60 ± 0.40	0.64 ± 0.38
rs6905288	226.5 ± 104.8	223.4 ± 102.6	245.9 ± 95.5	0.63 ± 0.40	0.63 ± 0.37	0.59 ± 0.37
rs9491696	233.8 ± 106.6	224.5 ± 105.8	222.5 ± 98.5	0.61 ± 0.38	0.63 ± 0.39	0.65 ± 0.39
rs1055144	226.4 ± 105.6	226.7 ± 103.2	226.1 ± 105.6	0.62 ± 0.39	0.63 ± 0.39	0.63 ± 0.38
rs718314	214.1 ± 101.2	228.2 ± 104.0	226.6 ± 104.9	0.67 ± 0.38	0.62 ± 0.38	0.64 ± 0.39
rs1443512	232.8 ± 110.3	221.7 ± 100.1	228.1 ± 106.0	0.59 ± 0.34	0.63 ± 0.37	0.63 ± 0.40
rs4823006	219.4 ± 100.1	224.1 ± 104.9	230.5 ± 104.7	0.60 ± 0.38	0.68 ± 0.41	0.59 ± 0.36

Abbreviation: BMI, body mass index; SFA, subcutaneous fat area; SNP, single nucleotide polymorphism; VFA, visceral fat area; V/S, ratio of viscera fat area to subcutaneous fat area.
11, allele 1/allele 1; 12, allele 1/allele 2; 22, allele 2/allele 2. Allele 1 and allele 2 of each SNP are indicated in Table 2.

Table 4. Relationship between fat distribution-associated loci and adiposity measures

SNP ID	Nearby gene	BMI			VFA			SFA			V/S		
		β	s.e.	*p*-value	β	s.e.	*p*-value	β	s.e.	*p*-value	β	s.e.	*p*-value
rs984222	*TBX15*	0.003	0.003	0.24	0.010	0.009	0.25	0.006	0.007	0.41	0.004	0.008	0.59
rs1011731	*DNM3*	−0.005	0.005	0.29	0.001	0.015	0.95	−0.009	0.012	0.43	0.010	0.013	0.43
rs4846567	*LYPLAL1*	−0.003	0.003	0.33	0.015	0.009	0.11	−0.010	0.007	0.16	0.025	0.008	**0.0020**
rs1659258	*THNSL2*	−0.003	0.004	0.39	−0.013	0.012	0.29	−0.007	0.010	0.47	−0.006	0.011	0.59
rs10195252	*GRB14*	0.001	0.005	0.86	0.003	0.016	0.83	0.010	0.013	0.44	−0.007	0.014	0.65
rs6784615	*NISCH*	0.019	0.011	0.087	0.073	0.035	**0.040**	0.000	0.028	1.00	0.073	0.031	**0.020**
rs6795735	*ADAMTS9*	−0.004	0.004	0.32	−0.005	0.012	0.71	0.005	0.010	0.60	−0.010	0.011	0.37
rs6861681	*CPEB4*	−0.010	0.005	**0.036**	−0.007	0.015	0.65	−0.018	0.012	0.13	0.011	0.013	0.39
rs1294421	*LY86*	0.008	0.004	**0.042**	0.001	0.012	0.94	0.012	0.010	0.20	−0.011	0.011	0.29
rs6905288	*VEGFA*	−0.001	0.003	0.75	−0.011	0.011	0.34	−0.004	0.009	0.63	−0.006	0.010	0.51
rs9491696	*RSPO3*	0.002	0.003	0.49	−0.003	0.009	0.72	0.011	0.007	0.13	−0.014	0.008	0.079
rs1055144	*NFE2L3*	0.000	0.003	0.93	0.007	0.009	0.46	0.002	0.007	0.73	0.004	0.008	0.60
rs718314	*ITPR2*	0.004	0.003	0.21	0.004	0.010	0.71	0.005	0.008	0.57	−0.001	0.009	0.93
rs1443512	*HOXC13*	0.001	0.004	0.80	−0.009	0.012	0.45	−0.001	0.009	0.94	−0.008	0.010	0.43
rs4823006	*ZNRF3*	−0.004	0.003	0.21	−0.004	0.009	0.64	−0.009	0.007	0.23	0.005	0.008	0.58

Abbreviation: BMI, body mass index; SFA, subcutaneous fat area; SNP, single nucleotide polymorphism; VFA, visceral fat area; V/S, ratio of viscera fat area to subcutaneous fat area.
Data were derived from linear regression analysis. BMI, VFA, SFA, and V/S values were logarithmically transformed. The logarithmically transformed BMI, VFA, and SFA values and the V/S ratio were adjusted for age and gender. Numbers in bold indicate a *p*-value of <0.05.

Table 5. Relationship between rs4846567 and adiposity in men and women

Phenotype	Gender	Values at each genotype			Additive model	
		GG	GT	TT	β (s.e.)	*p*-value
n	Men	239	296	97		
	Women	292	361	134		
BMI	Men	29.6 ± 5.1	30.2 ± 6.3	29.9 ± 7.0	−0.002 (0.004)	0.71
	Women	27.9 ± 4.9	28.4 ± 5.4	28.4 ± 5.6	−0.004 (0.004)	0.31
VFA	Men	153.6 ± 64.3	157.6 ± 70.2	143.9 ± 60.4	0.013 (0.012)	0.27
	Women	106.9 ± 56.5	100.1 ± 53.0	99.3 ± 51.0	0.015 (0.013)	0.25
SFA	Men	200.1 ± 106.8	213.3 ± 114.2	195.5 ± 92.2	−0.004 (0.012)	0.71
	Women	235.3 ± 93.2	244.0 ± 93.8	257.7 ± 115.6	−0.015 (0.009)	0.091
V/S	Men	0.89 ± 0.43	0.83 ± 0.39	0.83 ± 0.40	0.018 (0.011)	0.12
	Women	0.48 ± 0.28	0.43 ± 0.23	0.42 ± 0.26	0.029 (0.011)	**0.0078**

Abbreviation: BMI, body mass index; SFA, subcutaneous fat area; SNP, single nucleotide polymorphism; VFA, visceral fat area; V/S, ratio of viscera fat area to subcutaneous fat area.
Values are shown as the mean ± s.d. Data were derived from linear regression analysis. The BMI, VFA, SFA, and V/S values were logarithmically transformed. Logarithmically transformed BMI, VFA, and SFA values and the V/S ratio were adjusted for age and gender. Numbers in bold indicate a *p*-value of <0.05.

mentary Table 5).

Discussion

The most predictive risk factor for metabolic syndrome is the accumulation of visceral adipose tissue. Visceral fat mass measurement by CT is more precise than that derived from BMI, waist circumference measurement, or the waist-hip ratio. Furthermore, VFA is superior to waist circumference and the waist-hip ratio with regard to the prediction of metabolic risk factor clustering[19]. We found that the G-allele, which is rs4846567 in the *LYPLAL1* gene, was marginally associated with an increased V/S ratio. Patients possessing the G-allele of rs4846567 had an increased tendency toward VFA and a decreased tendency towards SFA, which resulted in increased V/S ratios. Fox *et al.*[14] recently reported the association between the G-allele of rs4846567 and the V/S ratio ($p=0.0002$); therefore, it is likely that rs4846567 has a strong influence on the V/S ratio.

We have previously reported that rs2605100 in the *LYPLAL1* gene was not associated with VFA or SFA in a sample of 1228 subjects drawn from the Japanese population (1211 of whom overlapped with this study)[13]. SNP rs2605100 was not significantly associated with VFA ($p=0.75$), SFA ($p=0.20$), or V/S ratio ($p=0.43$) in the current study. Although 2 SNPs are in the same linkage disequilibrium (LD) block according to the HapMap database, the values of D' (0.62) and r^2 (0.12) are relatively low. We have also reported that rs1558902 and rs1421085 in the *FTO* gene are significantly associated with SFA[13]. In this study, rs1558902 and rs1421085 were significantly associated with SFA ($p=0.00011$ and 0.00012, respectively), but not with VFA ($p=0.11$ and 0.092, respectively) or the V/S ratio ($p=0.096$ and 0.12, respectively). A recent report by Fox *et al.*[14] also indicated that rs1558902 was strongly associated with SFA ($p=6.2\times10^{-7}$), but not with VFA or the V/S ratio. *FTO* rs1558902 may thus have a stronger influence on the accumulation of subcutaneous fat than it does on the accumulation of visceral fat.

NISCH rs6784615 was nominally associated with increased VFA, but had no effect on SFA, and thus, the V/S ratio increased. *NISCH* rs6784615 was not associated with VFA or the V/S ratio in a recent report[14], nor did we find any association between SNPs in genes other than *LYPLAL1* and the VFA, SFA, and V/S ratio values in the current study.

We did not find a significant association between *THNSL2* rs1659258 and VFA or the V/S ratio in men and women. At some SNPs, risk allele frequencies in the present study were very different in this study from those in previous studies (**Supplementary Table 6**). Risk allele frequencies of *DNM3* rs1011731, *ADAMTS9* rs6795735, *CPEB4* rs6861681, and *LY86* rs1294421 in the Japanese (0.10-0.16) were much lower than those in the European population (0.34-0.57). Risk allele frequencies of *GRB14* rs10195252

Supplementary Table 1. Mean BMI, VFA, SFA, and V/S ratio of the 15 fat distribution risk variants in men and women

SNP ID	Mean ± s.d.					
	BMI (kg/m²)			VFA (cm²)		
Gender	11	12	22	11	12	22
rs984222						
Men	29.5 ± 6.6	30.4 ± 5.7	29.6 ± 6.1	153.5 ± 66.8	158.5 ± 67.1	149.3 ± 66.0
Women	29.1 ± 5.6	27.8 ± 5.0	28.3 ± 5.2	105.8 ± 52.6	100.3 ± 53.6	104.0 ± 55.4
rs1011731						
Men	30.1 ± 6.2	29.5 ± 5.2	27.9 ± 4.0	154.5 ± 67.6	153.6 ± 64.5	126.4 ± 22.6
Women	28.2 ± 5.2	28 ± 5.2	28.0 ± 4.6	102.6 ± 54.8	103.0 ± 49.8	102.7 ± 74.1
rs4846567						
Men	29.6 ± 5.1	30.2 ± 6.3	29.9 ± 7.0	153.6 ± 64.3	157.6 ± 70.2	143.9 ± 60.4
Women	27.9 ± 4.9	28.4 ± 5.4	28.4 ± 5.6	106.9 ± 56.5	100.1 ± 53.0	99.3 ± 51.0
rs1659258						
Men	29.8 ± 6.1	30.1 ± 5.7	32.0 ± 3.3	152.1 ± 67.0	159.2 ± 66.4	152.3 ± 57.0
Women	28.2 ± 5.3	28.0 ± 4.6	28.8 ± 7.9	102.1 ± 53.6	104.5 ± 54.2	100.5 ± 66.8
rs10195252						
Men	28.0 ± 1.6	29.7 ± 6.2	30.0 ± 6.0	165.7 ± 38.2	140.5 ± 56.3	156.3 ± 68.5
Women	29.2 ± 7.4	28.4 ± 5.4	28.2 ± 5.2	171.3 ± 130.4	103.2 ± 54.2	102.1 ± 53.0
rs6784615						
Men	-	27.9 ± 4.2	30.0 ± 6.0	-	141.2 ± 58.7	154.5 ± 66.9
Women	-	27.4 ± 5.4	28.2 ± 5.2	-	80.4 ± 48.9	103.1 ± 54.1
rs6795735						
Men	26.9 ± 3.9	29.7 ± 5.1	30.1 ± 6.3	122.2 ± 49.8	158.9 ± 69.2	153.2 ± 65.8
Women	27.3 ± 5.6	28.2 ± 5.5	28.2 ± 5.1	90.5 ± 52.2	104.9 ± 55.1	102.3 ± 53.8
rs6861681						
Men	33.4 ± 5.0	29.5 ± 6.6	30.0 ± 5.8	151.4 ± 76.9	164.9 ± 76.1	151.3 ± 63.9
Women	26.1 ± 3.8	27.5 ± 5.0	28.4 ± 5.3	105.8 ± 73.1	93.9 ± 46.6	104.6 ± 55.2
rs1294421						
Men	28.2 ± 4.5	31.1 ± 7.4	29.6 ± 5.4	153.4 ± 79.9	157.8 ± 71.0	152.9 ± 64.7
Women	28.4 ± 4.6	28.6 ± 5.8	28.0 ± 4.9	99.5 ± 51.3	100.2 ± 52.2	103.9 ± 55.0
rs6905288						
Men	30.2 ± 6.5	29.4 ± 5.2	30.5 ± 3.4	154.7 ± 67.4	151.5 ± 66.3	166.5 ± 60.9
Women	28.0 ± 5.0	28.4 ± 5.4	29.2 ± 6.4	101.6 ± 54.5	105.1 ± 53.8	97.1 ± 51.2
rs9491696						
Men	30.3 ± 6.5	29.7 ± 5.9	30.1 ± 5.6	153.7 ± 69.9	151.8 ± 62.6	159.6 ± 71.2
Women	28.4 ± 5.3	28.1 ± 5.1	28.1 ± 5.3	105.4 ± 55.0	100.5 ± 53.1	103.6 ± 55.1
rs1055144						
Men	30.4 ± 7.5	29.9 ± 5.2	29.7 ± 6.0	155.9 ± 76.6	155.2 ± 64.0	150.8 ± 63.6
Women	28.3 ± 4.8	27.9 ± 5.2	28.5 ± 5.6	99.4 ± 50.4	100.6 ± 51.7	108.8 ± 60.4
rs718314						
Men	29.5 ± 5.5	29.9 ± 5.5	30.0 ± 6.3	155.8 ± 54.8	153.0 ± 64.5	154.3 ± 69.3
Women	27.1 ± 4.7	28.0 ± 4.8	28.4 ± 5.6	95.6 ± 45.8	103.0 ± 56.3	103.2 ± 53.3
rs1443512						
Men	31.6 ± 8.4	29.6 ± 5.1	30.0 ± 6.2	149.4 ± 64.2	152.8 ± 66.5	154.6 ± 67.1
Women	28.2 ± 5.2	28.0 ± 4.7	28.3 ± 5.5	100.5 ± 55.3	100.5 ± 51.7	103.8 ± 55.1
rs4823006						
Men	28.5 ± 4.0	30.1 ± 5.9	30.1 ± 6.4	138.5 ± 54.4	160.7 ± 68.3	149.8 ± 66.5
Women	28.0 ± 5.6	28.1 ± 5.3	28.3 ± 5.0	102.0 ± 60.6	103.3 ± 54.2	102.3 ± 52.2

(Cont Supplementary Table 1)

SNP ID	Mean ± s.d.					
	SFA (cm^2)			V/S		
Gender	11	12	22	11	12	22
rs984222						
Men	200.6 ± 97.9	213.6 ± 115.7	197.7 ± 103.3	0.86 ± 0.46	0.87 ± 0.43	0.84 ± 0.35
Women	255.0 ± 101.7	233.1 ± 94.6	250.0 ± 98.6	0.44 ± 0.22	0.46 ± 0.27	0.44 ± 0.25
rs1011731						
Men	208.3 ± 110.9	195.7 ± 99.0	168.3 ± 59.3	0.85 ± 0.41	0.88 ± 0.40	0.82 ± 0.30
Women	243.7 ± 99.9	241.6 ± 88.1	235.1 ± 85.7	0.45 ± 0.26	0.46 ± 0.26	0.43 ± 0.21
rs4846567						
Men	200.1 ± 106.8	213.3 ± 114.2	195.5 ± 92.2	0.89 ± 0.43	0.83 ± 0.39	0.83 ± 0.40
Women	235.3 ± 93.2	244.0 ± 93.8	257.7 ± 115.6	0.48 ± 0.28	0.43 ± 0.23	0.42 ± 0.26
rs1659258						
Men	202.9 ± 106.7	210.7 ± 115.6	224.9 ± 80.9	0.85 ± 0.42	0.86 ± 0.36	0.75 ± 0.36
Women	243.3 ± 97.6	242.9 ± 93.9	252.0 ± 142.4	0.45 ± 0.25	0.46 ± 0.26	0.42 ± 0.24
rs10195252						
Men	185.8 ± 32.7	191.6 ± 97.0	208.1 ± 110.8	0.94 ± 0.36	0.84 ± 0.37	0.86 ± 0.41
Women	190.2 ± 81.4	247.7 ± 95.4	243.0 ± 98.2	0.97 ± 0.71	0.43 ± 0.21	0.45 ± 0.25
rs6784615						
Men	–	194.9 ± 95.3	206.1 ± 109.0	–	0.77 ± 0.29	0.86 ± 0.41
Women	–	235.4 ± 81.2	243.5 ± 98.1	–	0.34 ± 0.14	0.45 ± 0.26
rs6795735						
Men	156.6 ± 99.0	205.3 ± 103.2	206.5 ± 110.3	0.94 ± 0.37	0.87 ± 0.42	0.85 ± 0.40
Women	235.2 ± 120.3	251.9 ± 99.5	240.5 ± 96.1	0.40 ± 0.17	0.44 ± 0.24	0.46 ± 0.26
rs6861681						
Men	324.7 ± 104.8	197.9 ± 110.0	206.4 ± 107.6	0.45 ± 0.11	0.95 ± 0.50	0.83 ± 0.38
Women	221.1 ± 92.1	228.3 ± 86.5	247.1 ± 99.7	0.48 ± 0.25	0.44 ± 0.24	0.45 ± 0.26
rs1294421						
Men	193.4 ± 95.6	220.2 ± 125.3	201.0 ± 102.0	0.81 ± 0.25	0.85 ± 0.45	0.86 ± 0.39
Women	232.3 ± 82.1	252.8 ± 105.4	239.4 ± 94.1	0.46 ± 0.27	0.42 ± 0.22	0.46 ± 0.27
rs6905288						
Men	206.3 ± 110.3	198.3 ± 103.5	236.1 ± 79.8	0.86 ± 0.42	0.86 ± 0.38	0.74 ± 0.26
Women	243.0 ± 97.2	242.6 ± 97.9	253.9 ± 107.2	0.45 ± 0.25	0.46 ± 0.24	0.46 ± 0.40
rs9491696						
Men	207.4 ± 112.9	204.1 ± 110.7	206.1 ± 99.9	0.85 ± 0.43	0.85 ± 0.40	0.87 ± 0.39
Women	252.8 ± 97.7	242.0 ± 98.2	235.3 ± 95.7	0.44 ± 0.23	0.44 ± 0.25	0.48 ± 0.30
rs1055144						
Men	203.2 ± 111.7	209.2 ± 109.2	200.2 ± 104.8	0.86 ± 0.42	0.85 ± 0.40	0.86 ± 0.41
Women	243.8 ± 97.6	241.3 ± 95.6	246.6 ± 101.8	0.44 ± 0.25	0.45 ± 0.27	0.45 ± 0.23
rs718314						
Men	202.7 ± 110.5	210.9 ± 112.2	202.1 ± 105.6	0.89 ± 0.34	0.84 ± 0.42	0.86 ± 0.40
Women	223.7 ± 92.8	240.9 ± 95.6	247.5 ± 99.7	0.48 ± 0.30	0.45 ± 0.26	0.44 ± 0.25
rs1443512						
Men	202.4 ± 124.6	198.2 ± 99.8	208.8 ± 111.5	0.84 ± 0.31	0.86 ± 0.36	0.85 ± 0.43
Women	255.3 ± 94.6	241.3 ± 96.3	243.5 ± 98.8	0.40 ± 0.22	0.45 ± 0.27	0.45 ± 0.25
rs4823006						
Men	179.9 ± 76.3	208.0 ± 112.6	208.9 ± 109.4	0.85 ± 0.41	0.89 ± 0.40	0.81 ± 0.40
Women	243.2 ± 105.4	240.4 ± 94.0	245.7 ± 98.7	0.44 ± 0.26	0.46 ± 0.29	0.44 ± 0.23

Abbreviations: BMI, body mass index; SFA, subcutaneous fat area; SNP, single nucleotide polymorphism; VFA, visceral fat area; V/S, ratio of viscera fat area to subcutaneous fat area.
11, allele 1/allele 1; 12, allele 1/allele 2; 22, allele 2/allele 2. Allele 1 and allele 2 of each SNP are indicated in Table 2.

Supplementary Table 2. Relationship between fat distribution-associated loci and adiposity measures in men and women

SNP ID	Nearby gene	Gender	BMI			VFA			SFA			V/S		
			β	s.e.	*p*-value	β	s.e.	*p*-value	β	s.e.	*p*-value	β	s.e.	*p*-value
rs984222	*TBX15*	Men	0.002	0.004	0.65	0.016	0.012	0.20	0.014	0.012	0.25	0.002	0.011	0.89
		Women	0.004	0.004	0.26	0.005	0.013	0.67	0.000	0.009	0.96	0.006	0.011	0.59
rs1011731	*DNM3*	Men	−0.007	0.007	0.29	−0.009	0.019	0.62	−0.024	0.019	0.20	0.015	0.018	0.40
		Women	−0.003	0.006	0.64	0.010	0.022	0.65	0.004	0.015	0.78	0.006	0.019	0.75
rs4846567	*LYPLAL1*	Men	−0.002	0.004	0.71	0.013	0.012	0.27	−0.004	0.012	0.71	0.018	0.011	0.12
		Women	−0.004	0.004	0.31	0.015	0.013	0.25	−0.015	0.009	0.091	0.029	0.011	**0.0078**
rs1659258	*THNSL2*	Men	−0.008	0.006	0.17	−0.022	0.016	0.18	−0.015	0.016	0.35	−0.007	0.015	0.66
		Women	0.001	0.005	0.86	−0.003	0.018	0.85	0.001	0.012	0.95	−0.004	0.016	0.79
rs10195252	*GRB14*	Men	0.005	0.007	0.46	0.024	0.021	0.25	0.022	0.021	0.28	0.002	0.020	0.93
		Women	−0.003	0.007	0.63	−0.016	0.024	0.50	−0.002	0.016	0.92	−0.015	0.021	0.49
rs6784615	*NISCH*	Men	0.019	0.014	0.17	0.046	0.040	0.24	−0.013	0.039	0.74	0.060	0.037	0.11
		Women	0.015	0.019	0.43	0.097	0.063	0.12	0.013	0.042	0.76	0.084	0.054	0.12
rs6795735	*ADAMTS9*	Men	−0.005	0.006	0.38	0.001	0.017	0.97	−0.007	0.017	0.68	0.008	0.016	0.63
		Women	−0.003	0.005	0.60	−0.008	0.017	0.66	0.014	0.012	0.22	−0.022	0.015	0.14
rs6861681	*CPEB4*	Men	−0.005	0.007	0.47	0.027	0.020	0.18	−0.002	0.020	0.92	0.029	0.019	0.13
		Women	−0.014	0.006	0.028	−0.034	0.022	0.11	−0.031	0.014	0.030	−0.003	0.019	0.88
rs1294421	*LY86*	Men	0.009	0.006	0.12	0.008	0.017	0.62	0.012	0.017	0.48	−0.003	0.016	0.83
		Women	0.006	0.005	0.19	−0.005	0.017	0.77	0.012	0.011	0.28	−0.017	0.015	0.24
rs6905288	*VEGFA*	Men	0.003	0.005	0.55	−0.005	0.015	0.74	−0.011	0.015	0.44	0.006	0.014	0.64
		Women	−0.005	0.005	0.29	−0.018	0.016	0.27	0.000	0.011	0.97	−0.018	0.014	0.18
rs9491696	*RSPO3*	Men	0.002	0.004	0.58	−0.012	0.012	0.31	0.003	0.012	0.83	−0.015	0.011	0.19
		Women	0.002	0.004	0.57	0.006	0.013	0.65	0.018	0.008	**0.038**	−0.012	0.011	0.27
rs1055144	*NFE2L3*	Men	−0.003	0.004	0.55	−0.002	0.012	0.86	0.000	0.012	0.98	−0.002	0.011	0.87
		Women	0.002	0.004	0.69	0.013	0.013	0.30	0.005	0.009	0.60	0.009	0.011	0.43
rs718314	*ITPR2*	Men	0.000	0.005	0.95	−0.006	0.014	0.63	−0.010	0.013	0.45	0.004	0.013	0.78
		Women	0.007	0.004	0.11	0.012	0.015	0.42	0.016	0.010	0.097	−0.004	0.013	0.73
rs1443512	*HOXC13*	Men	0.004	0.005	0.48	−0.004	0.016	0.78	−0.007	0.015	0.63	0.003	0.015	0.83
		Women	−0.001	0.005	0.80	−0.011	0.017	0.51	0.005	0.011	0.64	−0.016	0.014	0.26
rs4823006	*ZNRF3*	Men	−0.005	0.005	0.30	0.004	0.013	0.76	−0.016	0.013	0.22	0.020	0.012	0.10
		Women	−0.003	0.004	0.45	−0.010	0.013	0.43	−0.004	0.009	0.63	−0.006	0.011	0.59

Abbreviations: BMI, body mass index; SFA, subcutaneous fat area; SNP, single nucleotide polymorphism; VFA, visceral fat area; V/S, ratio of viscera fat area to subcutaneous fat area.
Data were derived from linear regression analysis. BMI, VFA, SFA, and V/S values were logarithmically transformed. The logarithmically transformed BMI, VFA, and SFA values and the V/S ratio were adjusted for age. Numbers in bold indicate a *p*-value of <0.05.

Supplementary Table 3. Correlation of V/S ratio with various clinical parameters

Parameters	*r*	*p*-value
age	0.218	$<2.2\times10^{-16}$
Log_{10} (BMI)	0.002	0.94
Log_{10} (FPG)	0.193	2.5×10^{-13}
Log_{10} (Insulin)	0.111	5.6×10^{-5}
Log_{10} (HOMA-IR)	0.148	7.6×10^{-8}
Total cholesterol	0.043	0.10
Log_{10} (triglycerides)	0.372	$<2.2\times10^{-16}$
HDL-cholesterol	−0.282	$<2.2\times10^{-16}$
SBP	0.103	0.00026
DBP	0.149	1.1×10^{-7}

Abbreviations: DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL, high density lipoprotein; HOMA-IR; homeostasis model assessment-insulin resistance index; SBP, systolic blood pressure.
HOMA-IR was assessed as fasting insulin (μU/mL) × fasting plasma glucose/405. Spearman correlation coefficients were used to evaluate correlations of V/S ratio with various parameters. Values of V/S ratio, BMI, FPG, insulin, HOMA-IR, and triglycerides were logarithmically transformed.

and *VEGFA* rs6905288 in the Japanese (0.92 and 0.78) were much higher than those in the European population (0.60 and 0.56). The risk allele frequencies affected the power of the study and effect size. Thus, ethnic differences exist, although further studies with more subjects are required to investigate potential associations between the above-mentioned 15 SNPs and fat distribution in the Japanese population, and further investigation of others SNPs in the LD is also warranted.

Sexual dimorphism has long been acknowledged with regard to fat distribution[20]. *LYPLAL1* rs4846567 had a stronger effect on the V/S ratio in women ($p=0.0078$) than in men ($p=0.12$), although neither was significant after correction for multiple comparisons. This SNP has been previously reported to be significantly associated with the waist-hip ratio in women, but not men[12]. A recent report also indicated that rs4846567 was strongly associated with the V/S ratio in women ($p=0.0004$), but not men ($p=0.05$)[14]. We have recently reported that rs1004467 in the cytochrome P450, family 17, subfamily A, polypeptide 1 (*CYP17A1*) gene and rs11191548 in the 5'-nucleotidase, cytosolic II (*NT5C2*) gene were significantly associated with reductions in both VFA and SFA in women, but not in men[21]. Our studies and previous reports highlight the need for a better understanding of the underlying molecular mechanisms in the sex differences in the regulation of the distribution of body fat.

In addition to the rs4846567 genotype, fasting plasma glucose, triglycerides, and HDL-cholesterol reportedly each affect the V/S ratio independently[22]. Polymorphism rs4846567 exists in the downstream region of the *LYPLAL1* gene, encoding lysophospholipase-like protein. The crystal structure of *LYPLAL1* is closely related to acyl protein thioesterases, and *LYPLAL1* exhibits neither phospholipase nor triacylglycerol lipase activity, but accepts short-chain substrates[23]. Although the physiological substrates involved remain unclear, the *LYPLAL1* gene may contribute to fat distribution via involvement in lipid and/or glucose metabolism. Indeed, *LYPLAL1* mRNA expression is increased in the subcutaneous adipose tissue of obese subjects[24], but not in their visceral adipose tissue. Previous reports and our results suggest that the *LYPLAL1* gene plays a different role in the visceral and subcutaneous adipose tissue mass. Further investigation is necessary to elucidate the effect of rs4846567 on the expression of *LYPLAL1* and the role of the *LYPLAL1* gene in the regulation of fat distribu-

Supplementary Table 4. Multiple linear regression analysis for V/S ratio using SNP rs4846567 and other features as explanatory variables

Explanatory variable	β	s.e.	*p*-value
rs4846567 (2/1/0)	0.028	0.008	0.00029
Age (year)	0.007	0.000	$<2\times10^{-16}$
Gender (men/women, 1/0)	0.275	0.011	$<2\times10^{-16}$
Log_{10} (FPG) (mg/dL)	0.220	0.052	2.8×10^{-5}
Log_{10} (triglycerides) (mg/dL)	0.194	0.026	8.7×10^{-14}
HDL-cholesterol (mg/dL)	−0.001	0.000	0.00025
Adjusted R^2	0.45		

Abbreviations: FPG, fasting plasma glucose; HDL, high density lipoprotein; SNP, single-nucleotide polymorphism.
Data were derived from linear regression analysis. Values of V/S ratio, FPG, and triglycerides were logarithmically transformed.

Supplementary Table 5. The association between the fat distribution-susceptible SNPs and the metabolic traits

SNP ID	Nearby gene	FPG (mg/dL)		Insulin (μU/mL)		HOMA-IR		T. Chol. (mg/dL)	
		β (s.e.)	*p*-value	β (s.e.)	*p*-value	β (s.e.)	*p*-value	β (s.e.)	*p*-value
rs984222	*TBX15*	−0.004 (0.004)	0.35	0.012 (0.012)	0.29	0.010 (0.013)	0.45	0.001 (1.424)	1.00
rs1011731	*DNM3*	−0.011 (0.006)	0.082	0.033 (0.019)	0.095	0.024 (0.022)	0.28	2.310 (2.370)	0.33
rs4846567	*LYPLAL1*	−0.010 (0.004)	0.012	0.007 (0.012)	0.55	−0.003 (0.013)	0.84	0.414 (1.434)	0.77
rs1659258	*THNSL2*	−0.002 (0.005)	0.65	−0.014 (0.016)	0.40	−0.012 (0.018)	0.52	−3.402 (1.958)	0.083
rs10195252	*GRB14*	0.007 (0.007)	0.35	−0.001 (0.022)	0.95	0.002 (0.025)	0.94	−0.766 (2.604)	0.77
rs6784615	*NISCH*	−0.002 (0.016)	0.92	−0.041 (0.046)	0.37	−0.036 (0.053)	0.49	4.169 (5.697)	0.46
rs6795735	*ADAMTS9*	−0.003 (0.005)	0.61	−0.006 (0.016)	0.70	−0.006 (0.018)	0.75	2.125 (1.961)	0.28
rs6861681	*CPEB4*	0.005 (0.007)	0.49	−0.002 (0.020)	0.92	−0.001 (0.022)	0.98	−2.249 (2.391)	0.35
rs1294421	*LY86*	−0.01 (0.005)	0.052	0.006 (0.016)	0.72	0.003 (0.018)	0.87	−0.060 (1.934)	0.98
rs6905288	*VEGFA*	0.001 (0.005)	0.85	0.011 (0.014)	0.44	0.007 (0.016)	0.67	0.571 (1.759)	0.75
rs9491696	*RSPO3*	−0.007 (0.004)	0.069	−0.025 (0.012)	0.029	−0.032 (0.013)	0.013	−1.832 (1.408)	0.19
rs1055144	*NFE2L3*	0.002 (0.004)	0.69	0.006 (0.012)	0.61	0.010 (0.013)	0.46	1.892 (1.443)	0.19
rs718314	*ITPR2*	0.001 (0.004)	0.85	0.013 (0.013)	0.33	0.013 (0.015)	0.40	−2.407 (1.624)	0.14
rs1443512	*HOXC13*	0.001 (0.005)	0.82	−0.008 (0.015)	0.61	−0.008 (0.017)	0.63	0.983 (1.842)	0.59
rs4823006	*ZNRF3*	−0.006 (0.004)	0.16	0.010 (0.012)	0.41	0.006 (0.014)	0.65	−2.211 (1.479)	0.14

SNP ID	Nearby gene	Triglycerides (mg/dL)		HDL-C (mg/dL)		SBP (mmHg)		DBP (mmHg)	
		β (s.e.)	*p*-value	β (s.e.)	*p*-value	β (s.e.)	*p*-value	β (s.e.)	*p*-value
rs984222	*TBX15*	0.011 (0.009)	0.19	−0.735 (0.550)	0.18	−1.585 (0.664)	0.017	−0.536 (0.462)	0.25
rs1011731	*DNM3*	0.009 (0.014)	0.55	1.169 (0.917)	0.20	−1.655 (1.089)	0.13	−0.421 (0.758)	0.58
rs4846567	*LYPLAL1*	0.000 (0.009)	0.99	−0.145 (0.556)	0.79	−0.719 (0.670)	0.28	−0.351 (0.466)	0.45
rs1659258	*THNSL2*	−0.015 (0.012)	0.22	−0.048 (0.761)	0.95	−0.322 (0.910)	0.72	0.124 (0.632)	0.85
rs10195252	*GRB14*	0.004 (0.016)	0.79	0.662 (1.006)	0.51	0.255 (1.188)	0.83	−0.235 (0.826)	0.78
rs6784615	*NISCH*	0.033 (0.035)	0.35	−3.463 (2.197)	0.12	−1.653 (2.671)	0.54	−1.087 (1.857)	0.56
rs6795735	*ADAMTS9*	−0.008 (0.012)	0.49	1.118 (0.760)	0.14	0.998 (0.925)	0.28	−0.009 (0.643)	0.99
rs6861681	*CPEB4*	0.006 (0.015)	0.67	−0.558 (0.927)	0.55	0.735 (1.101)	0.51	0.260 (0.766)	0.73
rs1294421	*LY86*	−0.024 (0.012)	0.045	0.690 (0.751)	0.36	−1.441 (0.910)	0.11	−0.907 (0.634)	0.15
rs6905288	*VEGFA*	−0.015 (0.011)	0.16	0.817 (0.681)	0.23	0.953 (0.821)	0.25	1.363 (0.569)	**0.017**
rs9491696	*RSPO3*	−0.022 (0.009)	0.012	0.328 (0.546)	0.55	−0.080 (0.650)	0.90	−0.348 (0.452)	0.44
rs1055144	*NFE2L3*	0.002 (0.009)	0.84	0.052 (0.559)	0.93	−0.323 (0.675)	0.63	−0.370 (0.469)	0.43
rs718314	*ITPR2*	0.003 (0.010)	0.73	−0.875 (0.629)	0.16	0.739 (0.762)	0.33	0.731 (0.530)	0.17
rs1443512	*HOXC13*	−0.004 (0.011)	0.72	0.309 (0.721)	0.67	0.541 (0.848)	0.52	−0.833 (0.589)	0.16
rs4823006	*ZNRF3*	0.000 (0.009)	0.98	0.227 (0.574)	0.69	−0.806 (0.689)	0.24	−0.636 (0.479)	0.18

Abbreviations: DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high density lipoprotein cholesterol; HOMA-IR; homeostasis model assessment-insulin resistance index; SBP, systolic blood pressure; SNP, single-nucleotide polymorphism; T. Chol., total cholesterol.

HOMA-IR was assessed as fasting insulin (μU/mL) × fasting plasma glucose/405. Data were derived from linear regression analysis. Values of FPG, insulin, HOMA-IR, and triglycerides were logarithmically transformed. Each metabolic phenotype was adjusted for age, gender, and logarithmically transformed BMI. Numbers in bold indicate a *p*-value of < 0.05.

Supplementary Table 6. Risk allele frequencies

SNP ID	Chr	Position (Build 36.3)	Nearby gene	Risk allele	Risk allele frequency	
					This study	Previous reports*
rs984222	1	119,305,366	*TBX15*	G	0.39	0.37
rs1011731	1	170,613,171	*DNM3*	G	0.10	0.57
rs4846567	1	217,817,340	*LYPLAL1*	G	0.61	0.28
rs1659258	2	88,440,703	*THNSL2*	A	0.85	0.92
rs10195252	2	165,221,337	*GRB14*	T	0.92	0.60
rs6784615	3	52,481,466	*NISCH*	T	0.98	0.94
rs6795735	3	64,680,405	*ADAMTS9*	C	0.15	0.41
rs6861681	5	173,295,064	*CPEB4*	A	0.10	0.34
rs1294421	6	6,688,148	*LY86*	G	0.16	0.39
rs6905288	6	43,866,851	*VEGFA*	A	0.78	0.56
rs9491696	6	127,494,332	*RSPO3*	G	0.50	0.52
rs1055144	7	25,837,634	*NFE2L3*	T	0.53	0.21
rs718314	12	26,344,550	*ITPR2*	G	0.74	0.74
rs1443512	12	52,628,951	*HOXC13*	A	0.18	0.24
rs4823006	22	27,781,671	*ZNRF3*	A	0.33	0.57

Abbreviations: Chr, chromosome; SNP, single-nucleotide polymorphism. *The data are cited from Heid *et al.*[12], except rs1659258. The data of rs1659258 are cited from Fox *et al.*[14]

tion.

Some of the waist-hip ratio-susceptible SNPs investigated in the current study are reportedly associated with metabolic traits[12]; however, our multiple tests did not identify any significant associations between these SNPs and metabolic traits. These results were similar to those reported by Burgdorf *et al.*[25]; therefore, it is likely that each individual waist-hip ratio-susceptible polymorphism may exert only a weak effect on metabolic traits. The results observed in the current study were also inevitably influenced by the low power of this study. Further studies with more subjects are required to investigate the association between 15 SNPs and metabolic traits.

Conclusion

In summary, we showed that *LYPLAL1* rs4846567 is marginally associated with an increased V/S ratio. Our results and previous reports suggest that a region in close proximity to the *LYPLAL1* gene is involved in increasing the relative amount of visceral fat mass.

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Conflicts of Interest

None.

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Leptin restores the insulinotropic effect of exenatide in a mouse model of type 2 diabetes with increased adiposity induced by streptozotocin and high-fat diet

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[1]*Department of Diabetes, Endocrinology, and Nutrition, Kyoto University Graduate School of Medicine, Kyoto, Japan;* [2]*Medical Innovation Center, Kyoto University Graduate School of Medicine, Kyoto, Japan;* [3]*Institute for Advancement of Clinical and Translational Science, Kyoto University Hospital, Kyoto, Japan; and* [4]*Department of Human Health Science, Kyoto University Graduate School of Medicine, Kyoto, Japan*

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Sakai T, Kusakabe T, Ebihara K, Aotani D, Yamamoto-Kataoka S, Zhao M, Gumbilai VM, Ebihara C, Aizawa-Abe M, Yamamoto Y, Noguchi M, Fujikura J, Hosoda K, Inagaki N, Nakao K. Leptin restores the insulinotropic effect of exenatide in a mouse model of type 2 diabetes with increased adiposity induced by streptozotocin and high-fat diet. *Am J Physiol Endocrinol Metab* 307: E712–E719, 2014. First published August 26, 2014; doi:10.1152/ajpendo.00272.2014.—Leptin may reduce pancreatic lipid deposition, which increases with progression of obesity and can impair β-cell function. The insulinotropic effect of glucagon-like peptide-1 (GLP-1) and the efficacy of GLP-1 receptor agonist are reduced associated with impaired β-cell function. In this study, we examined whether leptin could restore the efficacy of exenatide, a GLP-1 receptor agonist, in type 2 diabetes with increased adiposity. We chronically administered leptin (500 $\mu g \cdot kg^{-1} \cdot day^{-1}$) and/or exenatide (20 $\mu g \cdot kg^{-1} \cdot day^{-1}$) for 2 wk in a mouse model of type 2 diabetes with increased adiposity induced by streptozotocin and high-fat diet (STZ/HFD mice). The STZ/HFD mice exhibited hyperglycemia, overweight, increased pancreatic triglyceride level, and reduced glucose-stimulated insulin secretion (GSIS); moreover, the insulinotropic effect of exenatide was reduced. However, leptin significantly reduced pancreatic triglyceride level, and adding leptin to exenatide (LEP/EX) remarkably enhanced GSIS. These results suggested that the leptin treatment restored the insulinotropic effect of exenatide in the mice. In addition, LEP/EX reduced food intake, body weight, and triglyceride levels in the skeletal muscle and liver, and corrected hyperglycemia to a greater extent than either monotherapy. The pair-feeding experiment indicated that the marked reduction of pancreatic triglyceride level and enhancement of GSIS by LEP/EX occurred via mechanisms other than calorie restriction. These results suggest that leptin treatment may restore the insulinotropic effect of exenatide associated with the reduction of pancreatic lipid deposition in type 2 diabetes with increased adiposity. Combination therapy with leptin and exenatide could be an effective treatment for patients with type 2 diabetes with increased adiposity.

drug therapy; combination; insulin secretion

LEPTIN, AN ADIPOCYTE-DERIVED hormone, has therapeutic potential for treating diabetes and obesity (7, 13 19, 27, 32, 34). In our previous clinical trial in patients with lipodystrophy (6), we confirmed the therapeutic usefulness of leptin as a glucose-lowering agent, and it was first approved for the treatment of lipodystrophy in Japan in March 2013. Given these glucoregulatory effects of leptin, we and others have reported the therapeutic usefulness of leptin for various forms of diabetes, including type 2 diabetes, in rodent models (20, 23, 26, 28, 47). The glucoregulatory effects of leptin are associated with the reduction of ectopic lipid deposition, which increases with progression of obesity (36, 39, 46). The reduction of ectopic lipid deposition in the liver and skeletal muscle could improve insulin sensitivity (42). In the pancreas, the reduction of ectopic lipid deposition could improve β-cell function such as glucose-stimulated insulin secretion (GSIS) in rodents and humans (22, 39, 46).

On the other hand, glucagon-like peptide-1 (GLP-1), a hormone released from the L cells of the intestine, improves glucose metabolism by enhancing GSIS (18). However, in patients with type 2 diabetes, the insulinotropic effect of GLP-1 is substantially reduced (15, 29). This reduction may be a consequence of the diabetic state rather than a contributor to it (30). Chronic hyperglycemia and hyperlipidemia could reduce β-cell function and could reduce the insulinotropic effect of GLP-1 (10, 15). The correction of both these abnormalities may restore β-cell function and may restore the insulinotropic effects of GLP-1 (11, 14, 49). Pancreatic lipid deposition can also reduce β-cell function (41, 45), but its effect on the insulinotropic effect of GLP-1 remains unknown.

In patients with type 2 diabetes, pancreatic lipid deposition increases with progression of obesity, and GSIS is reduced (17, 36, 41, 45). Therefore, we speculated that leptin could restore the insulinotropic effect of GLP-1 associated with the reduction of pancreatic lipid deposition and enhance the efficacy of GLP-1 receptor agonists. If this hypothesis is confirmed, we might be able to manage type 2 diabetes more effectively.

In the present study, we examined whether leptin could reduce pancreatic lipid deposition and enhance the insulinotropic effect of exenatide, a GLP-1 receptor agonist, in a mouse model of type 2 diabetes with increased adiposity induced by streptozotocin (STZ) and high-fat diet (HFD) (STZ/HFD mice) (20).

MATERIALS AND METHODS

Animals. Seven-week old male C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). The mice were individually caged and kept at a constant room temperature (25°C) under a 12:12-h light-dark cycle with ad libitum access to water and a standard diet

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(SD; NMF, 3.5 kcal/g, and 13% of energy as fat; Oriental Yeast, Tokyo, Japan). Animal care and all experiments were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Generation of the mouse model of type 2 diabetes with increased adiposity. We generated a mouse model of type 2 diabetes with increased adiposity, as described previously (20). Eight-week-old male C57BL/6J mice were intraperitoneally injected one time with STZ (120 μg/g body wt) to induce partial loss of pancreatic β-cells. Three weeks after the STZ injection, the mice that exhibited hyperglycemia (over 250 mg/dl, ad libitum) were fed with HFD (D12451, 4.7 kcal/g, and 45% of energy as fat; Research Diets, New Brunswick, NJ) for 5 wk and used for the infusion experiments from 16 wk of age. The mice continued to receive HFD during the infusion and pair-feeding (PF) experiments. Age-matched male C57BL/6J mice fed SD without an STZ injection were used as normal controls (NCs).

Leptin and/or exenatide infusion experiment. The STZ/HFD mice were divided into four infusion groups [saline alone (SAL), leptin alone (LEP), exenatide alone (EX), and leptin plus exenatide (LEP/EX)] to counterbalance their starting body weights and blood glucose levels. On *day 0*, all of the mice were implanted with two miniosmotic pumps subcutaneously in the midscapular region (Alzet model 2002; Alza, Palo Alto, CA). Each pump chronically delivered either saline, recombinant mouse leptin (500 $\mu g \cdot kg^{-1} \cdot day^{-1}$; Amgen, Thousand Oaks, CA), or exenatide (20 $\mu g \cdot kg^{-1} \cdot day^{-1}$; Bachem, Bubendorf, Switzerland) for 14 days. To examine the insulinotropic effect of exenatide in NCs, we also chronically administered exenatide (20 $\mu g \cdot kg^{-1} \cdot day^{-1}$) for 14 days as described above.

Food intake and body weight. The food intake and body weight of the mice were measured every day between 1500 and 1700 for 14 days.

Indirect calorimetry. The measurement of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) was performed 48 h between *day 9* and *10* after >72 h of acclimation using an Oxymax indirect calorimeter (Columbus Instruments, Columbus, OH). The respiratory exchange ratio [RER, ratio of CO_2 production to O_2 ($\dot{V}CO_2/\dot{V}O_2$)] was calculated and averaged across the measurement session.

Metabolic variables. Right before the infusion experiments, blood samples were obtained after 4 h of fasting. During the infusion experiments, ad libitum blood glucose levels were determined after tail bleeds using a reflectance glucometer by the glucose oxidase method between 1500 and 1700. At the end of the infusion experiment, blood was obtained from the inferior vena cava after 4 h of fasting. The plasma levels of insulin, leptin, triglyceride, total cholesterol, and nonesterified fatty acid (NEFA) were measured as described previously (20). The plasma exenatide levels were measured using ELISA kits specific for exenatide (Phoenix Pharmaceuticals, Burlingame, CA).

Insulin tolerance test and intraperitoneal glucose tolerance test. Either an insulin tolerance test (ITT) or intraperitoneal glucose tolerance test (IPGTT) was performed in each mouse on *day 11*. For ITTs, the mice were intraperitoneally injected with 0.4 mU/g human regular insulin (Humulin R; Eli Lilly Japan, Kobe, Japan) after 4 h of fasting. For IPGTTs, the mice were intraperitoneally injected with 1.0 mg/g glucose after overnight fasting. Blood samples were obtained from the tail vein at the indicated time points after insulin or glucose injection. GSIS was assessed by dividing the incremental insulin response by the incremental glucose response from 0 to 15 min [Δinsulin/Δglucose (0–15 min) (ng/ml ÷ mg/dl × 10^3)] during IPGTTs.

Pancreatic, liver, and skeletal muscle triglyceride levels and pancreatic insulin level. The pancreas, liver, and gastrocnemius muscles were isolated at the end of the experiments after 4 h of fasting. Tissue triglyceride and pancreatic insulin levels were measured as described previously (20, 28).

PF experiment. The STZ/HFD mice were randomly divided into three groups (SAL, LEP/EX, and PF) to counterbalance the starting body weights and blood glucose levels. The PF group was fed daily the same amount of HFD as that consumed by the LEP/EX group once at the end of the light phase for 14 days. Saline, leptin (500 $\mu g \cdot kg^{-1} \cdot day^{-1}$), and exenatide (20 $\mu g \cdot kg^{-1} \cdot day^{-1}$) were chronically infused in each group as described above for 14 days.

Data analyses. Data were expressed as means ± SE. In Figs. 1*A*, 1*D*, 1*E*, 1*F*, 3*A*, 3*B*, 4*C*, 4*D*, and 4*L*, comparisons were made using two-way repeated-measure ANOVA models for all the data. For within-group and between-group comparisons, corresponding contrasts were tested within the model. Between-group comparisons were made at all time points. In Table 1, comparisons were made using Student's *t*-test in each parameter. In the other figures and Table 2, comparisons were made using one-way ANOVA followed by Tukey's multiple-comparison test. A P value <0.05 was considered statistically significant.

RESULTS

Generation of the mouse model of type 2 diabetes with increased adiposity. As shown in Table 1, the STZ/HFD mice manifested hyperphagia and increased body weight. Hyperglycemia was exacerbated, although the plasma insulin levels were similar to those in NCs, suggesting the development of insulin resistance and impaired β-cell ability to secrete insulin adequately. In these mice, the plasma cholesterol and tissue triglyceride levels were also increased. Reduced pancreatic insulin levels in the STZ/HFD mice suggested substantial loss of pancreatic β-cells. GSIS was also reduced. These charac-

Table 1. *Metabolic characteristics of the mouse model of type 2 diabetes with increased adiposity*

Variables	Mouse Group	
	NC	STZ/HFD
Food intake, kcal/wk	82.1 ± 2.1	98.6 ± 3.4**
Body wt, g	25.9 ± 0.4	28.2 ± 0.5**
Leptin, ng/ml	2.4 ± 0.1	5.4 ± 0.3**
Blood glucose, mg/dl	124.9 ± 4.5	407.5 ± 30.6**
Insulin, ng/ml	0.97 ± 0.07	1.03 ± 0.06
Triglyceride, mg/dl	56.4 ± 2.4	57.3 ± 5.2
NEFA, meq/l	0.47 ± 0.03	0.56 ± 0.03
Total cholesterol, mg/dl	43.7 ± 3.8	93.6 ± 4.3**
Muscle triglyceride level, mg/g tissue	4.9 ± 1.5	11.1 ± 2.2*
Liver triglyceride level, mg/g tissue	10.0 ± 1.1	30.7 ± 3.3**
Pancreatic triglyceride level, mg/g tissue	5.7 ± 1.0	24.1 ± 5.2**
Pancreatic insulin level, ng/mg tissue	519.4 ± 22.4	28.4 ± 4.2**
GSIS [Δinsulin/Δglucose (0–15 min)], ng/ml ÷ mg/dl × 10^3	1.2 ± 0.2	−0.1 ± 0.2**
GSIS under exenatide infusion [Δinsulin/Δglucose (0–15 min)], ng/ml ÷ mg/dl × 10^3	3.2 ± 0.9	0.4 ± 0.5*

Data are reported as means ± SE. NC, normal chow; STZ, streptozotocin; HFD, high-fat diet; NEFA, nonesterified fatty acid. Parameters except for food intake, tissue triglyceride levels, pancreatic insulin levels, and glucose-stimulated insulin secretion (GSIS) were measured right before the infusion experiment. Food intake was measured during 14 days of the saline infusion and halved; tissue triglyceride levels and pancreatic insulin levels were measured after the 14 days of saline infusion (n = 8 mice in each group). GSIS and GSIS under exenatide infusion were assessed as described in detail in MATERIALS AND METHODS (n = 5–6 mice in each group). *P < 0.05 and **P < 0.01 vs. NC.

Table 2. *Plasma leptin and exenatide levels in leptin- and/or exenatide-infused STZ/HFD mice*

Variables	Infusion Group			
	SAL	LEP	EX	LEP/EX
Leptin, ng/ml	8.6 ± 1.3	38.5 ± 5.1**††	5.2 ± 0.3	24.0 ± 4.4*#††
Exenatide, pmol/l	ND	ND	286.0 ± 78.9	235.7 ± 31.4

Data are reported as means ± SE; n = 12–14 mice in each group for leptin and n = 4–5 mice in each group for exenatide. SAL, slaine alone; LEP, leptin alone; EX, exenatide alone. Plasma samples were obtained on *day 14* of the infusion experiment. *P < 0.05 and **P < 0.01 vs. SAL. #P < 0.05 vs. LEP. ††P < 0.01 vs. EX. ND, not detected.

teristics were compatible with human type 2 diabetes with increased adiposity.

Effects of leptin and/or exenatide on glucose metabolism in the STZ/HFD mice. Continuous administration of leptin (500 $\mu g \cdot kg^{-1} \cdot day^{-1}$) and exenatide (20 $\mu g \cdot kg^{-1} \cdot day^{-1}$) elevated plasma leptin levels to almost 20–30 ng/ml above baseline and plasma exenatide levels to around 250 pmol/l, respectively, in the STZ/HFD mice (Table 2).

After the infusions, LEP, EX, and LEP/EX significantly corrected hyperglycemia. Furthermore, LEP/EX corrected hyperglycemia to a greater extent than either monotherapy (Fig. 1, *A* and *B*). Plasma insulin levels were not significantly different among each infusion group and the NC group (Fig. 1*C*), suggesting that factors affecting insulin secretion, such as blood glucose and lipid levels, insulin sensitivity, and other insulin secretagogues, worked differently in each group. Following this, we performed ITT to evaluate the effects on insulin sensitivity, the results of which showed a marked decrease in blood glucose levels in the LEP/EX group than in the other groups (Fig. 1*D*). Next, we performed IPGTT to evaluate the effects on insulin secretion, the results of which showed significant improvement of glucose tolerance in the LEP/EX group than in the other three infusion groups (Fig. 1*E*). The plasma insulin levels in the SAL group at 15 min did not increase from those at 0 min (Fig. 1*F*), and Δinsulin/Δglucose (0–15 min) values were severely reduced compared with those in the NC group (Fig. 1*G*), indicating reduced GSIS in the STZ/HFD mice (Table 1). In addition, GSIS under exenatide infusion, the Δinsulin/Δglucose (0–15 min) values in the exenatide (20 $\mu g \cdot kg^{-1} \cdot day^{-1}$)-infused mice, were markedly reduced in the STZ/HFD mice compared with those in the NC group (Table 1), suggesting a reduced insulinotropic effect

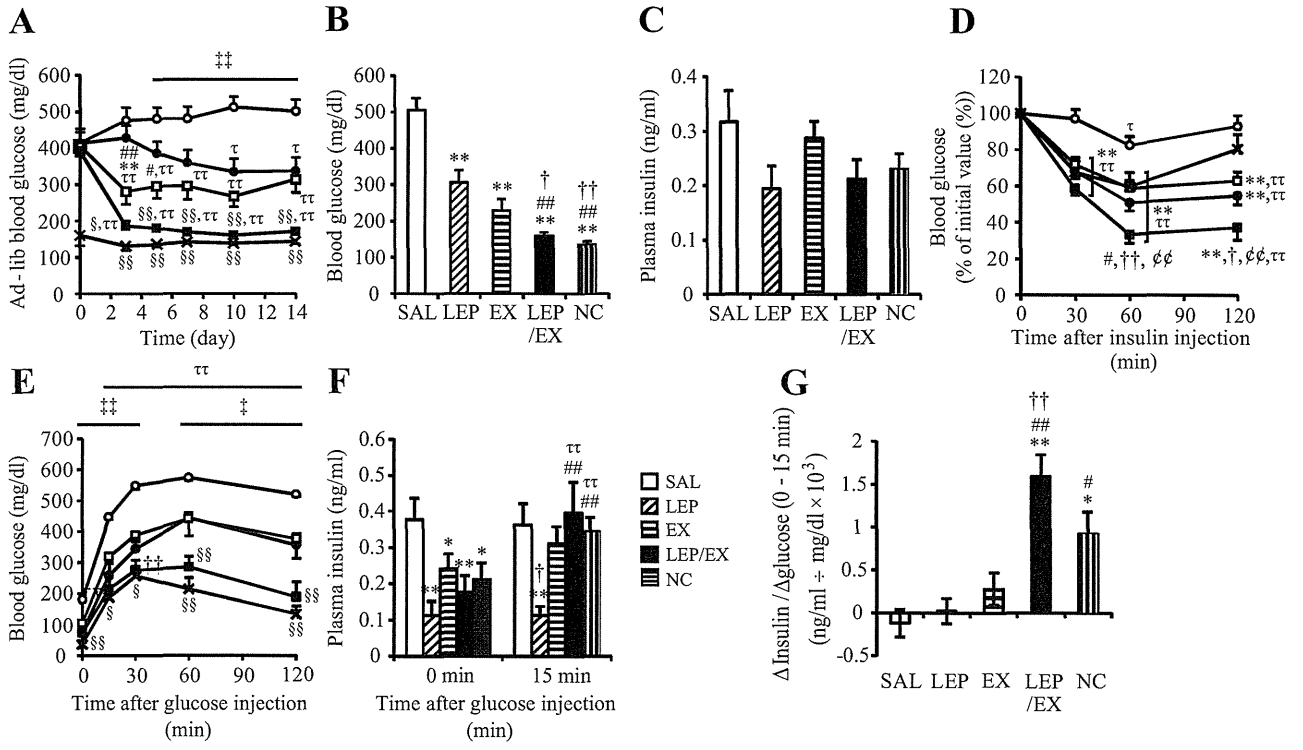

Fig. 1. Effects of leptin and/or exenatide on glucose metabolism in the streptozotocin (STZ)/high-fat diet (HFD) mice. *A*: ad libitum blood glucose levels in the saline alone (SAL, white circles), leptin alone (LEP, black circles), exenatide alone (EX, white squares), LEP/EX (black squares), and normal control (NC, cross marks) groups for 14 days (n = 14–18 mice in each infusion group; n = 8 mice in NC). *B* and *C*: plasma glucose levels (*B*) and insulin levels (*C*) on *day 14* (n = 14–18 in each group). *D*: %decrease of initial value of blood glucose levels during the insulin tolerance test (ITT) in the same four infusion groups and NC group on *day 11* (n = 8–10 in each group). *E*: blood glucose levels during the intraperitoneal glucose tolerance test (IPGTT, n = 8–10 in each group). *F*: plasma insulin levels at 0 and 15 min during IPGTT (n = 8–10 in each group). *G*: Δinsulin/Δglucose (0–15 min) values in IPGTT (n = 8–10 in each group). Data are reported as means ± SE. Between-group significant differences are indicated at each time point. *P < 0.05 and **P < 0.01 vs. SAL. #P < 0.05 and ##P < 0.01 vs. LEP. †P < 0.05 and ††P < 0.01 vs. EX. ¢¢P < 0.01 vs. NC. §P < 0.05 and §§P < 0.01 vs. SAL, LEP, and EX. ‡P < 0.05 and ‡‡P < 0.01 for SAL vs. the others. τP < 0.05 and ττP < 0.01 vs. the value at *day 0* or 0 min in the same infusion group.