Hypertension susceptibility genes on chromosome 2p24-p25 in a general Japanese population

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Background Genome-wide scans from Italy and China suggest a hypertension-susceptible locus between D2S2278 (nucleotides 11 245 080–11 245 358) and D2S168 (nucleotides 11 467 214–11 467 422) on chromosome 2.

Methods We performed a large association study of polymorphisms in this region with blood pressure modulation in a Japanese general population. Forty-seven polymorphisms in 14 genes between nucleotide 8 845 292 and nucleotide 11 946 689, which contains D2S2278 and D2S168, were genotyped in 1880 individuals, 796 of whom were hypertensive and 1084 normotensive.

Results Multivariate logistic regression analysis with adjustment for age, body mass index, presence of hyperlipidemia, diabetes mellitus, and current smoking and drinking revealed that one single nucleotide polymorphism (SNP), IMS-JST126186, in *HPCAL1* (hippocalcin-like 1) in women and two SNPs, IMS-JST149391 and IMS-JST149390, in *GREB1* (gene regulated by estrogen in breast cancer 1) in men were significantly associated with both prevalence of hypertension and blood pressure levels. To examine the role of *GREB1* in more detail, we identified 38 additional genetic variations in *GREB1* by direct sequencing, and eight polymorphisms were genotyped. One SNP, 45718A>G, was significantly associated with hypertension and blood pressure level in men, and this SNP was in linkage disequilibrium with a SNP present at the 3′ splice site of intron 11.

Conclusion Our study suggests that *GREB1* and *HPCAL1* are candidate hypertension-susceptibility genes in the Japanese general population and supports previous studies that also identified hypertension-related loci in this narrow region. *J Hypertens* 23:955–960 © 2005 Lippincott Williams & Wilkins.

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Introduction

Essential hypertension (EHT) is one of the common and important risk factors for stroke, coronary heart diseases and renal failure. It is a multi-factorial disorder that results from the interaction of a number of susceptibility genes and environmental factors [1]. The identification of genes that confer susceptibility to EHT has been the focus of intensive studies, which have generally taken one of two approaches; a search for candidate genes or genome-wide scans [2].

Genome-wide scans have revealed that all human chromosomes except 13 and 20 had blood pressure, hypertension or pre-eclampsia loci [3–6]. One of the most promising regions is on chromosome 2. Zhu *et al.* performed a genome-wide scan using Chinese sib-pairs and reported linkage of hypertension to chromosome 2q14-q23 [7]. Another genome-wide scan in an isolated population in Italy identified a hypertension susceptibility locus between D2S2278 and D2S168 in 2p24-25 [8]. A study of the full genomes of 15 Finnish families with pre-eclampsia found significant linkage between this condition and two loci, chromosome 2p25 near marker D2S168 and 9p13 [9]. Caulfield *et al.* also reported linkage of hypertension to chromosome 2q in 1599 severely hypertensive families [5].

In Japan, we have the JSNP database of a large number of single nucleotide polymorphisms (SNPs) from the general Japanese population [10]. To identify genes for hypertension susceptibility in the Japanese population, we undertook a detailed association study using the JSNP database, and focused on the region between D2S2278 and D2S168 in 2p24-25 (length 222 kb), which has already been linked to hypertension in a relatively isolated population [8].

Methods

Subjects of population study

The selection criteria and design of the Suita study have been described previously [11,12]. Leukocyte DNA was collected from participants between April 2002 and February 2003. Only those who gave written informed consent for genetic analyses were included in this study. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. The genotypes of 1880 samples (866 men including 456 normotensives and 410 hypertensives; 1014 women including 628 normotensives and 386 hypertensives) were determined. Routine blood examinations that included total serum cholesterol, high-density lipoprotein-cholesterol, triglyceride, and glucose levels were performed. Lifestyle (current smoking and alcohol drinking habits) and present illness (hypertension, hyperlipidemia, and diabetes) were collected.

Blood pressure was measured after at least 10 min of rest in a sitting position. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were the means of two measurements by well-trained doctors (recorded > 3 min apart). Hypertension was defined as SBP $\geq$ 140 mmHg, DBP $\geq$ 90 mmHg, or the current use of antihypertensive medication. Diabetes mellitus was defined as fasting plasma glucose $\geq$ 7.0 mmol/l (126 mg/dl) or non-fasting plasma glucose $\geq$ 11.1 mmol/l (200 mg/dl) or anti-diabetic medication or HbA1c $\geq$ 6.5%. Hyperlipidemia was defined as total cholesterol $\geq$ 5.68 mmol/l (220 mg/dl) or antihyperlipidemia medication. The body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared.

Genotyping of polymorphisms located between D2S2278 and D2S168 on chromosome 2

For this large-scale association study, the region between D2S2278 (nucleotides 11 245 080–11 245 358, NT_005334.14, build 34.3) and D2S168 (nucleotides 11 467 214–11 467 422) on chromosome 2 [8] was selected because this region is narrow enough for a SNP association study. Therefore, we genotyped SNPs in genes in the region located between D2S2278 and D2S168 and in the region just outside of these two markers. In the interval between nucleotide 8 845 292 and nucleotide 11 946 689, we selected 47 SNPs in 14 genes from the JSNP database (http://snp.ims.u-tokyo.ac.jp/) for a large-scale genotyping by the TaqMan-polymerase chain reaction method, as described previously [13]. The SNPs genotyped were as follows; IMS-JST063570, IMS-JST053874, IMS-JST072894, IMS-JST149078, IMS-JST116225, IMS-JST028894, IMS-JST028226, IMS-JST149098, IMS-JST149096, IMS-JST149087, IMS-JST060753, IMS-JST024762, IMS-JST024765, IMS-JST126186, IMS-JST126184, IMS-JST126167, IMS-JST126159, IMS-JST009517, IMS-JST073362, IMS-JST085615, IMS-JST175884, IMS-JST037805, IMS-JST085610, IMS-JST085609, IMS-JST064688, IMS-JST064690, IMS-JST085605, IMS-JST066564, IMS-JST042012, IMS-JST108315, IMS-JST108314, IMS-JST042011, IMS-JST126143, IMS-JST126142, IMS-JST066563, IMS-JST116274, IMS-JST116273, IMS-JST116270, IMS-JST116268, IMS-JST061384, IMS-JST061385, IMS-JST149391, IMS-JST149390, IMS-JST149403, IMS-JST025848, IMS-JST025849, IMS-JST153824. Thus, 14 genes, *ID2*, *ADAM17*, *TAF1B*, *LBP-32*, *HPCAL1*, *LOC130063*, *FLJ14075*, *ATP6V1C2*, *P5*, *KCNF1*, *MGC33602*, *ROCK2*, *GREB1*, and *LPIN1*, have been genotyped in this study.

Direct sequencing for SNP discovery of *GREB1*

A part, not all, of the *GREB1* (gene regulated by estrogen in beast cancer 1) gene has been sequenced in detail for SNP discovery, and those SNPs have been deposited in the JSNP database (http://snp.ims.u-tokyo.ac.jp/). We sequenced the rest of exons of *GREB1*, including exons 1, 3, 5–8, 10–14, 16–19, 21, 22, and 35–37, in this study. For DNA sequencing, 48 Japanese patients with EHT were recruited. The method of direct sequencing was described previously [14,15]. Thirty-eight SNPs were identified by sequencing. The sequence results of the *GREB1* gene are available on request. Twelve representative SNPs with a minor allele frequency of greater than 5% were genotyped by the TaqMan–polymerase chain reaction method [16], with the final successful genotyping of eight SNPs (−22923G>A, −13945A>T, 5921A>C, 29168G>A, 31080A>C, 39565G>A, 45718A>G, 81444G>A — the A of ATG of the initiator Met codon is denoted nucleotide +1).

Statistical analysis

Analysis of variance was used to compare mean values between groups, and if overall significance was demonstrated the intergroup difference was assessed by means of a general linear model. Frequencies were compared by chi-squared test.

Association of genetic models with blood pressures were performed through logistic regression analysis considering potential confounding risk variables, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (current smoking and drinking), and antihypertensive medication by sex. For multivariate risk predictors, the adjusted odds ratios were given with the 95% confidence intervals. The relationship between genotype and risk of hypertension was expressed in terms of odds ratios adjusted for possible confounding factors, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (current smoking and drinking) by sex. SAS statistical software (release 8.2; SAS Institute Inc., Cary, North Carolina, USA) was used

Table 1 Baseline characteristics of subjects in the Suita study

Variable	Men			Women		
	Total	Normotensive	Hypertensive	Total	Normotensive	Hypertensive
Number	866	456	410	1014	628	386
Age (years)	66.3 ± 11.0*	64.0 ± 11.7	68.8 ± 9.7**	63.3 ± 11.0	60.2 ± 11.7	68.4 ± 9.4**
Body mass index (kg/m^2)	23.2 ± 3.0*	22.7 ± 2.8	23.8 ± 3.0**	22.3 ± 3.2	21.8 ± 2.9	23.2 ± 3.3**
Systolic blood pressure (mmHg)	131.8 ± 19.4*	119.1 ± 11.9	145.7 ± 16.2**	128.1 ± 19.7	117.0 ± 12.2	146.0 ± 16.1**
Diastolic blood pressure (mmHg)	79.7 ± 10.7*	74.8 ± 8.7	85.3 ± 9.9**	76.6 ± 9.8	73.1 ± 8.3	82.3 ± 9.5**
Prevalence of hypertension (%)	47.3†	–	–	38.1	–	–
Fasting plasma glucose (mmol/l)	5.72 ± 1.45*	5.56 ± 1.33	5.90 ± 1.55**	5.29 ± 0.93	5.16 ± 0.80	5.50 ± 0.31**
Prevalence of diabetes (%)	11.1†	9.4	12.0	4.3	2.7	6.9††
Total cholesterol (mmol/l)	5.11 ± 0.79	5.14 ± 0.80	5.10 ± 0.76	5.57 ± 0.79*	5.51 ± 0.78	5.68 ± 0.79**
Triglyceride (mmol/l)	1.36 ± 1.05*	1.32 ± 1.18	1.41 ± 0.88	1.06 ± 0.56	0.99 ± 0.58	1.16 ± 0.49**
HDL-cholesterol (mmol/l)	1.42 ± 0.37	1.43 ± 0.36	1.42 ± 0.38	1.66 ± 0.40*	1.70 ± 0.40**	1.61 ± 0.38
Prevalence of hyperlipidemia (%)	25.9	25.9	25.8	54.5†	46.8	65.4††
Current smoker (%)	29.9†	33.8††	25.6	6.3	7.8††	3.8
Current alcohol drinker (%)	67.1†	62.1	73.2††	29.5	31.3	26.4

Values are mean ± standard deviation or percentage. HDL, high-density lipoprotein. Hypertension indicates systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg or antihypertensive medication; hyperlipidemia, total cholesterol ≥ 5.68 mmol/l (220 mg/dl) or antihyperlipidemia medication; diabetes, fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl) or non-fasting plasma glucose ≥ 11.1 mmol/l (200 mg/dl) or HbA1c ≥ 6.5% or anti-diabetic medication. *$P < 0.05$ between women and men, **$P < 0.05$ between normotension and hypertension by Student's t test, †$P < 0.05$ between women and men, ††$P < 0.05$ between normotension and hypertension by the chi-squared test.

for all analyses. For each pair of SNPs, the pairwise linkage disequilibrium parameters D' and r^2 were calculated on the basis of the genotyping data using the SNPAlyze version 3.1Pro (DYNACOM Co., Ltd., Mobara, Japan).

Results

Baseline characteristics of subjects in the Suita Study

The characteristics of the 1880 participants (866 men and 1014 women) are summarized in Table 1. Age, SBP, DBP, BMI, percentage of current smokers, percentage of current drinkers, and prevalence of hypertension and diabetes mellitus were significantly higher in men than in women. Total cholesterol, high-density lipoprotein-cholesterol, and percentage of hyperlipidemia were significantly higher in women than in men (Table 1).

Genotyping of polymorphisms located between D2S2278 and D2S168 on chromosome 2

The region between nucleotide 8 845 292 and nucleotide 11 946 689, which includes D2S2278 and D2S168, contained 36 genes including genes related to vascular function such as *ADAM17* (tumor necrosis factor-α converting enzyme), *KCNF1* (potassium voltage-gated channel subfamily F, member 1), and *ROCK2* (Rho-associated, coiled-coil containing protein kinase 2). We genotyped 47 SNPs in 14 genes in this region in 1880 Japanese individuals (hypertensive, $n = 796$; normotensive, $n = 1084$). The pairwise linkage disequilibrium parameter, D', calculated with the genotyped data indicated that this region has a haplotype-block structure, and 47 SNPs genotyped in the study have been represented by 22 independent SNPs with $D' > 0.98$.

Multivariate logistic regression analysis with adjustment for age, BMI, hyperlipidemia, diabetes mellitus, smoking, drinking, and antihypertensive medication identified 11 SNPs in three genes that were associated with hypertension using a dominant or recessive model ($P < 0.05$); IMS-JST024762, IMS-JST024765, IMS-JST126186 in *HPCAL1* (hippocalcin-like 1), IMS-JST085615 in *FLJ14075*, and IMS-JST116274, IMS-JST116273, IMS-JST116270, IMS-JST116268, IMS-JST149391, IMS-JST149390, and IMS-JST149403 in *GREB1* (Table 2). Among them, three SNPs (IMS-JST024762, IMS-JST024765, IMS-JST126186) in *HPCAL1*, four SNPs (IMS-JST116274, IMS-JST116273, IMS-JST116270, IMS-JST116268) in *GREB1* and two SNPs (IMS-JST149391, IMS-JST149390) in *GREB1* were separately in linkage disequilibrium ($D' > 0.98$). One of the SNPs, IMS-JST116268 in *GREB1*, showed a strong positive association with the presence of hypertension in men (CC + CT versus TT; odds ratio = 1.69, 95% confidence interval = 1.17–2.43, $P = 0.005$).

The SBP in women with the AA + AC genotype of the positively-associated SNP IMS-JST126186 in *HPCAL1* was 16.7 mmHg higher than that with the CC genotype ($P = 0.003$). The SBP in men with the GG + GC genotype of IMS-JST149391 in *GREB1* was 9.2 mmHg higher than in the men with the CC genotype ($P = 0.008$), and was 9.2 mmHg higher in men with the AA + AG genotype of IMS-JST149390 in *GREB1* than in those with the GG genotype ($P = 0.008$). These two *GREB1* SNPs were in tight linkage disequilibrium, as already mentioned.

These results suggest that three SNPs, IMS-JST126186 in *HPCAL1* and IMS-JST149391 and IMS-JST149390 in *GREB1*, are significantly associated with susceptibility to hypertension and blood pressure modulation.

Polymorphisms in *GREB1*

The *GREB1* gene consists of 37 exons and is 108 671 base pairs in size, starting at nucleotide 11 695 981 and ending at nucleotide 11 804 651 (NT_005334.14, build 34.3). It produces three alternative

Table 2 Single nucleotide polymorphisms (SNPs) showing significant association with hypertension among 47 SNPs in 14 genes on chromosome 2[a]

Gene	SNP	Allele 1/2 (allele frequency)	Sex	Genotype group	Odds ratio (95% CI)	P
HPCAL1	IMS-JST024762	C/T (0.734/0.266)	Women	CC CT + TT	1 0.75 (0.56–1.00)	0.050
HPCAL1	IMS-JST024765	G/A (0.385/0.615)	Women	GG GA + AA	1 1.59 (1.06–2.40)	0.026
HPCAL1	IMS-JST126186	A/C (0.880/0.120)	Women	AA + AC CC	1 0.17 (0.03–0.93)	0.040
			Men	AA + AC CC	1 0.39 (0.16–0.96)	0.041
FLJ14075	IMS-JST085615	C/T (0.640/0.360)	Men	CC CT + TT	1 1.43 (1.07–1.92)	0.015
GREB1	IMS-JST116274	A/G (0.346/0.654)	Women	AA AG + GG	1 1.65 (1.09–2.49)	0.019
GREB1	IMS-JST116273	G/A (0.810/0.190)	Men	GG GA + AA	1 0.70 (0.52–0.94)	0.017
GREB1	IMS-JST116270	G/T (0.690/0.310)	Women	GG + GT TT	1 0.57(0.36–0.90)	0.015
GREB1	IMS-JST116268	C/T (0.542/0.458)	Men	CC + CT TT	1 1.69 (1.17–2.43)	0.005
GREB1	IMS-JST149391	G/C (0.805/0.195)	Men	GG + GC CC	1 2.59 (1.03–6.48)	0.043
GREB1	IMS-JST149390	A/G (0.803/0.197)	Men	AA + AG GG	1 2.61 (1.04–6.53)	0.041
GREB1	IMS-JST149403	C/T (0.497/0.503)	Men	CC CT + TT	1 0.69 (0.49–0.98)	0.037

[a]Logistic regression analysis, adjusted for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (current smoking and drinking). CI, confidence interval.

transcripts, variants a–c. The SNPs in *GREB1*, which were deposited in the JSNP database, were obtained from a part of the coding region of *GREB1* and 20 exons remained to be sequenced. To identify polymorphisms in these regions, we sequenced 96 alleles from 48 hypertensives in exons 1, 3, 5–8, 10–14, 16–19, 21, 22, 35–37, and identified 38 additional polymorphisms. We identified seven missense mutations in *GREB1* that may affect protein function. We selected 12 SNPs for genotyping based on the presence of linkage disequilibrium ($r^2 > 0.5$) and minor allele frequency (> 5%), and successfully genotyped eight of these.

Polymorphisms that confer susceptibility to hypertension

Multivariate logistic regression analysis adjusting for the same factors as already described revealed that −13945A>T (AA versus AT + TT; odds ratio = 0.44, 95% confidence interval = 0.24–0.78, $P = 0.006$) and 45718A>G (AA versus AG + GG; odds ratio = 1.54, 95% confidence interval = 1.10–2.15, $P = 0.011$) in *GREB1*, which were identified by direct sequencing, were significantly associated with hypertension in men (Table 3). When the controls were defined as SBP ≤ 120 mmHg, DBP ≤ 80 mmHg, or no hypertensive medication, and hypertension was defined as SBP ≥ 160 mmHg, DBP ≥ 100 mmHg, or the current use of antihypertensive medication, 45718A>G was significantly associated with hypertension in men (AA versus AG + GG; odds ratio = 2.38, 95% confidence interval = 1.39–4.17, $P = 0.002$).

Table 3 Allele frequency and odds ratio of the presence of hypertension by genotypes of *GREB1* polymorphisms in men[a]

SNP (allele frequency)	Genotype group	Odds ratio (95% CI)	P
−13945A>T (0.246/0.754)	AA AT + TT	1 0.44 (0.24–0.78)	0.006
45718A>G (0.512/0.488)	AA AG + GG	1 1.54 (1.10–2.15)	0.011

[a]Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). CI, confidence interval.

The SNP 45718A>G in intron 20 was in linkage disequilibrium ($r^2 > 0.5$) with another 11 SNPs, one of which was 30767G>A. This SNP is present at the −1 position of the 3′ splice site of intron 11 that would disturb the consensus GT–AG rule for the proper splicing event, and would lead to aberrant splicing of exon 12, an exon specific for variant c transcripts of *GREB1* and not for variants a and b. These results suggest that 30767G>A in *GREB1* is also a hypertension/blood pressure modulation locus.

Discussion

Two genome scans for EHT, one in an Italian population and another in a Chinese population, identified a hypertension susceptibility locus near D2S168 on chromosome 2 [7,8]. Here, we conducted a cross-sectional study of hypertension and blood pressure modulation using a Japanese general population and found that the *HPCAL1* and *GREB1* genes are strong candidates for susceptibility to hypertension in the population. Although the association of individual SNPs is at best marginally significant given the number of tests performed, the association with multiple SNPs in *GREB1* suggests that *GREB1* plays a

role in blood pressure regulation that affects hypertension.

HPCAL1 encodes hippocalcin-like 1 protein. Hippocalcin is a Ca^{2+}-binding protein with three EF-hand structures, which are dominantly expressed in the hippocampal pyramidal layer [17]. It functions as a neuronal calcium sensor, and possesses a Ca^{2+}/myristoyl switch allowing it to translocate to membranes. Hippocalcin protects neurons against calcium-induced death stimuli in cooperation with neuronal apoptosis inhibitory protein [18]. Hippocalcin-like 1 protein and hippocalcin share 94% amino acid identity (182 out of 193 identical amino acid residues), suggesting similar function. The neuronal network that includes the hypothalamus and medulla oblongata in the brain plays an important role in the progression of hypertension [19], although the specific contribution of hippocalcin or *HPCAL1* to hypertension remains unclear.

GREB1 was isolated as an estrogen-responsive gene that was differentially expressed in β-estradiol-stimulated human breast carcinoma cells [20]. *GREB1* is an early gene in the estrogen receptor-regulated pathway and is thought to play an important role in hormone-responsive tissues and cancer. In experiments, estrogen has depressor effects through the improvement of endothelial dysfunction [21] and modulation of sympathetic nerve activation [22]. Estrogen insufficiency may be related to postmenoposal hypertension [23], although the use of hormone replacement therapy to prevent cardiovascular diseases remains controversial [24]. Genetic variation in estrogen receptor α has been associated with coronary artery disease and coronary artery wall atherosclerosis [25,26]. So it is possible that *GREB1* is related to vascular function via estrogen.

GREB1 generates three transcripts, variants a–c, with divergent 5′ and 3′ untranslated regions. GREB1a, GREB1b, and GREB1c proteins are 1949, 457, and 409 amino acids in length, respectively, with 396 common amino-terminal residues [20]. The SNP 45718A>G that was associated with hypertension and blood pressure modulation is in tight linkage disequilibrium with 30767G>A, which is located at the 3′ splice site of intron 11 (IVS11-1G>A). Therefore, it is likely to cause aberrant splicing of exon 12, which is specific to variant c transcripts and not variants a and b. Thus, 30767G>A may affect the normal splicing of *GREB1*, giving rise to disruption of c transcripts, which may explain the function of 45718A>G.

Chromosome 2 was already known to contain one or more hypertension susceptibility gene(s) [5,7–9], which may include the sodium bicarbonate cotransporter gene, *SLC4A5* [27]. In that study, 82 SNPs in eight genes between 40 and 140 cM on chromosome 2 were genotyped, and SNPs in the *GREB1* and *HPCAL1* genes, both of which are quite distal to *SLC4A5*, were found to be positively associated with hypertension in a Japanese population, suggesting that chromosome 2 may carry multiple hypertension susceptibility loci.

Study limitations

Since association studies are not consistently reproducible due to false-positives, false-negatives or true variability in association between different populations [28], the association of *GREB1* and *HPCAL1* polymorphisms to hypertension and/or blood pressure modulation must be re-examined in other populations.

Furthermore, the precise pathophysiological functions of both GREB1 and hippocalcin-like 1 proteins in blood pressure regulation need to be elucidated.

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β_2- and β_3-Adrenergic Receptor Polymorphisms Are Related to the Onset of Weight Gain and Blood Pressure Elevation Over 5 Years

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Background—The genes responsible for obesity are candidate genes for obesity-related diseases, such as hypertension. Functional polymorphisms in the β_2- and β_3-adrenergic receptors have been reported to be associated with hypertension and obesity.

Methods and Results—To longitudinally clarify the relevance to alterations in β-adrenergic receptor polymorphisms related to weight gain, blood pressure (BP) elevation, and sympathetic nerve activity as measured by plasma norepinephrine level, we studied 160 young, nonobese, normotensive men. Changes in body weight, BP, plasma norepinephrine levels, and β_2-adrenergic (Arg16Gly, Gln27Glu) and β_3-adrenergic (Trp64Arg) receptor polymorphisms were measured periodically over a 5-year period. Weight gain and BP elevation were defined as $\geq$10% increases from entry levels over 5 years in body mass index or mean BP. The presence of the Gly16 allele of Arg16Gly was associated with a higher frequency of weight gain and BP elevation over the 5-year period. The subjects carrying the Glu27 allele of Gln27Glu and the Trp64 allele of Trp64Arg had a higher frequency of BP elevation. Significantly higher levels of plasma norepinephrine at entry and at year 5 were observed in the subjects with the Gly16 allele of Arg16Gly and the Glu27 allele of Gln27Glu compared with those without the Gly16 or the Glu27 alleles.

Conclusions—These results demonstrate that the Gly16 allele is related to greater weight gain and BP elevation. Additionally, Glu27 and Trp64 alleles are linked to BP elevation. The subjects carrying the β_2-polymorphisms linked to weight gain and BP elevation also have higher plasma norepinephrine levels that are present at entry before weight gain and BP elevation. These findings suggest that β_2-adrenergic receptor polymorphisms in association with a heightened sympathetic nerve activity could predict the future onset of obesity and hypertension, as shown in the 5-year longitudinal study. (***Circulation.* 2005;111:3429-3434.**)

Key Words: hypertension ■ norepinephrine ■ obesity

Obesity and obesity-related cardiovascular disease are a rapidly growing public health problem,[1] and there is evidence that human obesity and hypertension have strong genetic as well as environmental determinants.[2–4] Reduced energy expenditure and resting metabolic rate are predictive of weight gain, and the sympathetic nervous system participates in regulating energy balance through thermogenesis. The thermogenic effects in obesity have been mainly attributed to the activity of the β_1- and β_2-adrenergic receptors in humans. However, reports of an association of β_2-adrenergic receptor polymorphisms with hypertension and obesity have been discordant.[5–7] Several observations have shown that the Trp64Arg polymorphism of the β_3-adrenergic receptor gene can also be associated with obesity[8–10]; however, this finding has not been confirmed in other studies.[11,12] Few studies have simultaneously taken into account obesity and hypertension as related to polymorphisms of β-adrenoceptor genes in the same study population followed longitudinally for several years. Additionally, plasma norepinephrine levels, as an index of sympathetic nerve activity (SNA), are also included in the present study.

Thus, this study examines the associations of polymorphisms of β-adrenergic receptors with plasma norepinephrine level (index of SNA), weight gain (obesity), and blood pressure (BP) elevation (hypertension) over 5 years in 160 subjects who at entry were young, nonobese, and normotensive.

Methods

Subjects

Subjects were recruited from a cohort of 1121 men who work in a single company in Osaka, Japan, as part of their annual medical

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TABLE 1. Polymorphism Genotypic Frequencies in Subjects With Significant Weight Gain (≥10%) and Mean BP Elevation (≥10%) Over 5 Years

	Genotypes			χ^2 Test for 3 Genotypes	χ^2 Test for Alleles
Arg16Gly of β_2-adrenoceptor gene	**Arg16/Arg16**	**Arg16/Gly16**	**Gly16/Gly16**		
Frequency					
With weight gain (n=59)	9 (15.3)	33 (55.9)	17 (28.8)	χ^2=7.98, P=0.019	χ^2=6.31, P=0.012
Without weight gain (n=101)	36 (35.6)	46 (45.5)	19 (18.8)		
With BP elevation (n=41)	4 (9.8)	20 (48.8)	17 (41.4)	χ^2=15.43, P<0.001	χ^2=14.42, P<0.001
Without BP elevation (n=119)	41 (34.5)	59 (49.6)	19 (16.0)		
Gln27Glu of β_2-adrenoceptor gene	**Gln27/Gln27**	**Gln27/Glu27**	**Glu27/Glu27**		
Frequency					
With weight gain (n=59)	50 (84.7)	9 (15.3)	0 (0.0)	...	χ^2=2.89, P=0.089
Without weight gain (n=92)	87 (94.6)	5 (5.4)	0 (0.0)		
With BP elevation (n=41)	32 (78.0)	9 (22.0)	0 (0.0)	...	χ^2=8.36, P=0.004
Without BP elevation (n=110)	105 (95.5)	5 (4.5)	0 (0.0)		
Trp64Arg of β_3-adrenoceptor gene	**Trp64/Trp64**	**Trp64/Arg64**	**Arg64/Arg64**		
Frequency					
With weight gain (n=59)	46 (78.0)	11 (18.6)	2 (3.4)	χ^2=7.41, P=0.025	χ^2=2.39, P=0.122
Without weight gain (n=99)	60 (60.6)	38 (38.4)	1 (1.0)		
With BP elevation (n=41)	35 (85.4)	5 (12.2)	1 (2.4)	χ^2=9.16, P=0.010	χ^2=5.25, P=0.022
Without BP elevation (n=117)	71 (60.7)	44 (37.6)	2 (1.7)		

Values in parentheses are percentage of subjects.

evaluation. Subjects at study entry were excluded who were aged >50 years, had obesity (body mass index [BMI] ≥25 kg/m^2),[13,14] had diabetes mellitus (fasting glucose level >100 mg/dL), and had hypertension (≥140/90 mm Hg). We also excluded subjects who were taking medication for hypertension, hyperlipidemia, hyperuricemia, or other illness. Only subjects who had steady body weight (weight had not changed significantly [<5%] over the past year before the entry period) were enrolled in this study.[15,16] After exclusion, 160 young, nonobese (BMI <25 kg/m^2), normotensive (<140/90 mm Hg) men on no medications were enrolled in the present study. Informed consent was obtained from each subject, as approved by the Ethics Committee of Osaka University Graduate School of Medicine, Osaka, Japan.

Measurements

After an overnight fast of >12 hours, BMI, total body fat mass, ratio of waist circumference to hip circumference (waist-to-hip ratio), BP, heart rate, venous sampling for plasma norepinephrine, and extraction of genomic DNA from leukocytes were taken every year for 5 years. BP and heart rate were measured with the subject in the recumbent position with an automated sphygmomanometer (TM-2713, A&D) with an adjusted cuff size, which had been standardized against a mercury sphygmomanometer. The percent body fat mass was determined with impedance measurements (BF-102, Tanita), and total body fat mass (kg) was calculated according to the following formula: (percent body fat mass/100)×body weight (kg).

Laboratory Determinations

Plasma norepinephrine was measured by high-performance liquid chromatography with a fluorometric method as previously described for this laboratory[17] (intra-assay coefficient of variation=2.1%; interassay coefficient of variation=3.6%; sensitivity=0.06 to 120 nmol/L).

Genotyping

Genotyping was performed by the TaqMan assay as previously described.[18] Two polymorphisms in the β_2-adrenergic receptors (arginine/glycine substitution, Arg16Gly; glutamine/glutamate substitution, Gln27Glu) of the β_2-adrenoceptor gene were studied.[6] One polymorphism (tryptophan/arginine substitution, Trp64Arg) of the β_3-adrenoceptor gene was also studied.[19,20] The probes and primers used in the TaqMan assay were as follows. For single-nucleotide polymorphisms in the β_2-adrenergic receptor gene, the probes and primers were as follows: for Arg16Gly, the probes were CGCATGGCTTCCATTGGGTGC and CGCATGGCTTCTATTGGGTGC, and the primers were GGAACGGCAGCGCCTTCT and CAGGACGATGAGAGACATGACGAT; for Gln27Glu, the probes were CTCGTCCCTTTCCTGCGTGACGT and CTCGTCCCTTTGCTGCGTGACGT (the primers used in this assay were the same as those used for Arg16Gly). For the Trp64Arg single-nucleotide polymorphism in the β_3-adrenergic receptors, the probes were TCTCGGAGTCCAGGCGATGGCCA and CTCGGAGTCCGGGCGATGGCC, and the primers were GGAGGCAACCTGCTGGTCAT and CACGAACACGTTGGTCATGGT.

Statistical Analysis

Genotype frequencies and the Hardy-Weinberg equilibrium were estimated with χ^2 test. Values are shown as mean±SD. All data analyses were performed with SPSS 8.0 for Windows programs. Changes in measured parameters within each group and differences among groups were examined by 2-way ANOVA. When these differences were significant, the Dunnett test was used to determine whether the differences of the mean measured variables at entry and 5 years were significant within the groups and among the groups compared from baseline. Values of P<0.05 were considered significant.

Results

Significant weight gain and BP elevation over 5 years were defined as a ≥10% increase in BMI or mean BP compared with values at entry.[16,21] Fifty-nine subjects had significant weight gain over 5 years, and 41 subjects had significant BP elevation. Table 1 shows the prevalence of weight gain and BP elevation at year 5. No subjects with the Glu27/Glu27 polymorphism of the β_2-adrenoceptor were detected in the present study. The allele frequency of Glu27 of the β_2-

TABLE 2. Characteristics of Subjects at Entry and at Year 5

	Subjects With Weight Gain		Subjects Without Weight Gain		Subjects With BP Elevation		Subjects Without BP Elevation	
	At Entry	At Year 5	At Entry	At Year 5	At Entry	At Year 5	At Entry	At Year 5
Subjects, n	59	59	101	101	41	41	119	119
Smoker/nonsmoker, n	15/44	10/49	22/79	13/88	13/28	8/33	24/95	15/104
Age, y	39±4	44±4‖	40±5	45±5‖	40±4	45±4‖	40±5	45±5‖
BMI, kg/m^2	22.2±1.8	24.6±2.0*§	22.9±1.7	22.4±1.9	22.6±1.6	24.3±2.1†§	22.7±1.7	22.8±1.9
Waist-to-hip ratio	0.92±0.11*	0.97±0.13*§	0.88±0.12	0.91±0.13	0.92±0.09†	0.94±0.11	0.89±0.11	0.92±0.12
Total fat mass, kg	14.1±2.1*	15.6±2.2*§	12.5±1.9	12.9±2.0	13.7±2.0†	15.1±2.0†§	12.8±2.0	13.4±2.2
Systolic BP, mm Hg	127±6	141±8*‖	128±6	131±7	132±7†	146±9‡‖	126±6	131±7
Diastolic BP, mm Hg	77±5*	80±5*	74±5	75±5	74±5	83±6†‖	76±4	75±6
Mean BP, mm Hg	94±5	101±6*§	93±5	94±6	93±6	104±6‡‖	92±6	94±6
Heart rate, bpm	70±5*	72±5	66±5	71±5§	71±4†	73±5	66±5	71±6§
Plasma norepinephrine, pmol/mL	1.18±0.11*	1.41±0.21*§	1.00±0.20	1.26±0.19	1.14±0.12†	1.43±0.24†§	1.01±0.16	1.27±0.18§

Data are mean±SD; n=160.
*$P<0.05$ vs subjects without weight gain; †$P<0.05$, ‡$P<0.01$ vs subjects without BP elevation; §$P<0.05$, ‖$P<0.01$ vs values at entry.

adrenoceptor polymorphism was 4.6%, and that of Arg64 of the β_3-adrenoceptor polymorphism was 17.4%, but all studied loci allele and genotype frequencies were in accordance with the Hardy-Weinberg equilibrium. The frequency distributions for homozygosity for the Arg16 and Gly16 alleles in this study were 28.1% and 22.5%. The frequency distributions for homozygosity for the Gln27 and Glu27 were 90.7% and 0.0%, and the frequency distributions for the Trp64 and Arg64 were 67.1% and 1.9%. The frequency distributions for homozygosity for the Glu27 and the Arg64 in our subjects were similar to those in previous studies in Japanese cohorts but lower than those found in studies in white subjects.[5,6,9,10,22,23] The frequency of Gly16 allele of the β_2-adrenoceptor gene was greater in subjects with weight gain than in those without weight gain. Additionally, the frequency of the Gly16 allele of the β_2-adrenergic receptor gene was significantly greater in subjects who showed a significant BP elevation over 5 years. The frequencies of the Glu27 and Trp64 alleles were higher in subjects with BP elevation than in those without BP elevation (Table 1).

Furthermore, to evaluate the relationships between the β-adrenoceptor alleles and weight gain–related BP elevation, we compared the frequencies of alleles between the groups with and without BP elevation in subjects who significantly gained body weight versus those without weight gain. In subjects who had a significant weight gain, those who also had a significant BP elevation carried a higher frequency of the Gly16 and Glu27 alleles compared with those without a significant BP elevation ($\chi^2=4.73$, $P=0.030$; $\chi^2=6.35$, $P=0.012$, respectively). In subjects who did not gain weight over the 5-year period, the allele frequencies in the 3 genotypes that were studied were similar in subjects with and without a BP elevation over time.

Table 2 shows the demographic characteristics of the 2 groups subdivided by significant weight gain (≥10%) over 5 years or BP elevation at entry and at year 5. At both periods, waist-to-hip ratio, total fat mass, heart rate, and plasma norepinephrine were higher in the group who had significant weight gain and in the group who had a significant rise in mean BP compared with those without weight gain or BP elevation. It is important to note that at entry, BMI and BP were similar between the groups with and without weight gain and BP elevation. Among the 41 subjects with a significant BP elevation, 32 of these individuals also had a significant weight gain.

Subjects were divided into the 2 subgroups in each studied genotype according to the dominant allele. Characteristics between those with and without the dominant allele are shown in Tables 3, 4, and 5. Total body fat mass and waist-to-hip ratio at entry in the subjects carrying the Gly16 allele and Glu allele of the β_2-adrenoceptor gene were greater than in the other genotypes (Tables 3 and 4). BMI and total body fat mass increased significantly in the subjects with the Gly16 allele and Glu27 allele of the β_2-adrenoceptor genes. Subjects who had the Gly16 and Glu27 of the β_2-adrenoceptor gene and the Trp64 of the β_3-adrenoceptor gene had significant increments in mean BP over the 5 years (Tables 3, 4, and 5).

As we have previously reported,[15,16,21] subjects with the most significant weight gain and BP elevation had the highest levels of plasma norepinephrine at entry compared with subjects without weight gain or BP elevation (Table 2). Plasma norepinephrine levels at both entry and year 5 were greater in subjects carrying Gly16 allele and Glu27 allele of the β_2-adrenoceptor genes than in the other genotypes (Tables 3 and 4). Plasma norepinephrine levels increased significantly over the 5-year period in those subjects with the abnormal β-adrenoceptor alleles. The same subjects also had higher plasma norepinephrine levels at entry.

Discussion

The present study shows that the Arg16Gly and the Gln27Glu of the β_2-adrenoceptor and the Trp64Arg of the β_3-adrenoceptor polymorphisms have a substantial influence on future gain in body weight or BP elevation in male subjects who were originally nonobese and normotensive. The subjects carrying the polymorphism for the Gly16, Glu27, and Trp64 alleles show higher frequency in those who had a

TABLE 3. Characteristics of Subjects According to Genotype of Arg16Gly at Entry and at 5 Years

	Without Gly16 Allele (Arg16Arg)		With Gly16 Allele (Arg16Gly+Gly16Gly)	
	At Entry	At Year 5	At Entry	At Year 5
Subjects, n	45	45	115	115
Smoker/nonsmoker, n (%)	10/35 (22.2/77.8)	7/38 (15.6/84.4)	27/88 (23.5/76.5)	16/99 (13.9/86.1)
BMI, kg/m^2	22.6±1.9	22.4±2.8	22.7±1.8	23.5±2.1*‡
Waist-to-hip ratio	0.87±0.10	0.88±0.11	0.91±0.10‡	0.95±0.12*‡
Total fat mass, kg	12.8±2.0	13.2±1.9	13.4±1.9‡	14.1±2.0*‡
Systolic BP, mm Hg	128±5	129±6	127±5	137±7†§
Diastolic BP, mm Hg	73±7	75±5	76±6	79±5‡
Mean BP, mm Hg	92±6	93±5	94±6	98±7*‡
Heart rate, bpm	65±6	69±5	69±6	72±6
Norepinephrine, pmol/mL	0.99±0.16	1.10±0.22	1.09±0.14‡	1.40±0.10†§

Data are mean±SD; n=160.
*P<0.05, †P<0.01 vs value at entry; ‡P<0.05, §P<0.01 vs subjects without Gly16 allele (Arg16Arg genotype).

significant weight gain or BP elevation over the 5-year study. Higher levels of plasma norepinephrine at entry were also seen in the groups with the Gly16 or Glu27 alleles. As we have shown in all studies, a heightened SNA (high mean plasma norepinephrine) predicted subsequent weight gain and BP elevation.[15,16,21] Now we show that the increased SNA is in part determined by the genetic influence of the β_2-adrenergic receptor systems.

Pathophysiological involvement of genetic abnormalities in the β_2-adrenergic receptor system in hypertension and obesity are well described.[5,6,24–26] Among β_2-adrenergic receptor polymorphisms, Arg16Gly and Gln27Glu are considered the most functionally important.[5,6,24–26] Gratze et al[27] found that young normotensive white men homozygous for the Gly16 allele of the β_2-adrenoceptor gene had higher BP and lower peripheral vasodilation after infusion of the β-blocker salbutamol. The Gly16 substitution exaggerates agonist-mediated receptor downregulation.[6,28] Our findings that the Gly16 allele is associated with weight gain and BP elevation associated with higher plasma norepinephrine levels are in accordance with these findings. The subjects who had weight gain–related BP elevation also had higher frequencies of the Gly16 and Glu27 alleles compared with those without BP elevation, suggesting that Gly16/Glu27 is related to obesity-related hypertension. On the other hand, the frequency associations of the Arg16 or Gly16 alleles of the Arg16Gly and the Gln27 or Glu27 alleles of the Gln27Glu with the onset of hypertension and obesity are more controversial.[6] The Glu27 receptor had been shown to be resistant to downregulation compared with Gln27 but when coexpressed with Arg16.[29] We were not able to observe any significant association of the Arg16 and Glu27 alleles with weight gain or BP elevation, probably because of the small sample size of the study.

The β_3-adrenergic receptor system is important in mediating the stimulation of lipolysis by catecholamines in white adipose cells in humans and in the development of obesity.[8–10] It is well documented that weight gain leads to BP elevation,[1,15,16] but there are few investigations about the genetic relations in the β_3-adrenoceptor such as polymor-

TABLE 4. Characteristics of Subjects According to Genotype of Gln27Glu at Entry and at 5 Years

	Without Glu27 Allele (Gln27Gln)		With Glu27 Allele (Gln27Glu)	
	At Entry	At Year 5	At Entry	At Year 5
Subjects, n	137	137	14	14
Smoker/nonsmoker, n (%)	34/103 (24.8/75.2)	22/115 (16.1/83.9)	3/11 (21.4/78.6)	1/13 (7.1/92.9)
BMI, kg/m^2	22.6±1.7	23.0±2.5	23.5±2.1	24.6±3.0*‡
Waist-to-hip ratio	0.89±0.10	0.92±0.11	0.92±0.11	0.99±0.10*‡
Total fat mass, kg	13.0±1.9	13.4±2.0	13.9±1.3‡	14.9±2.3*§
Systolic BP, mm Hg	127±5	135±5*	132±5	138±6*
Diastolic BP, mm Hg	75±5	76±6	77±6	83±5*§
Mean BP, mm Hg	93±5	94±5	95±5	101±6*§
Heart rate, bpm	67±5	71±6	69±5	70±6
Norepinephrine, pmol/mL	1.03±0.20	1.30±0.18*	1.29±0.14‡	1.42±0.19*‡

Data are mean±SD; n=151.
*P<0.05, †P<0.01 vs value at entry; ‡P<0.05, §P<0.01 vs subjects without Glu allele (Gln27Gln genotype).

TABLE 5. Characteristics of Subjects According to Genotype of Trp64Arg at Entry and at 5 Years

	With Trp64 Allele (Trp64Trp+Trp64Arg)		Without Trp64 Allele (Arg64Arg)	
Genotype	At Entry	At Year 5	At Entry	At Year 5
Subjects, n	155	155	3	3
Smoker/nonsmoker, n (%)	36/119 (23.2/76.8)	23/132 (14.6/85.4)	1/2 (33.3/66.7)	0/3 (0.0/100.0)
BMI, kg/m^2	23.1±1.7	23.2±2.7	22.8±0.5	24.0±0.6
Waist-to-hip ratio	0.90±0.06	0.93±0.08	0.90±0.09	0.94±0.10
Total fat mass, kg	13.2±1.8	13.9±2.0	13.1±2.0	13.5±2.0
Systolic BP, mm Hg	127±5	134±6†	126±6	128±7
Diastolic BP, mm Hg	75±5	78±5*	75±5	77±6
Mean BP, mm Hg	93±5	97±5†	92±5	94±6
Heart rate, bpm	68±5	71±6*	67±5	68±6
Norepinephrine, pmol/mL	1.06±0.20	1.31±0.14*	1.03±0.25	1.27±0.23

Data are mean±SD; n=158.
*P<0.05, †P<0.01 vs value at entry.

phisms in Trp64Arg and the association of these polymorphisms with hypertension in obesity.[30] Fujisawa et al[23] have shown in a Japanese population that the allele frequency of Arg64 in hypertensive subjects was similar to that in normotensive subjects. Other investigators have reported in a large Japanese cohort (n=3706) that the subjects with the Arg64/Arg64 genotype had a greater BMI and percent fat mass than those with the in Trp64/Trp64 genotype.[9] Conversely, we did not observe these associations in the genotype of the β_3-adrenoceptor in relation to weight gain–related BP elevation.

In the present study we used plasma norepinephrine levels as an index of SNA. Tuck,[31] Grassi and Esler,[32] and Rahn et al[33] observed that there are different results in SNA values in hypertensive patients depending on the method of SNA measurement, including regional norepinephrine spillover, muscle sympathetic nerve activity (microneurography), and plasma norepinephrine measurements. Spillover methods are considered the "gold standard" for SNA measurements, but in humans these are difficult and invasive measurements. Plasma norepinephrine levels are more practical for large population studies and represent several different processes (secretion, clearance, and reuptake).[3,15,16]

It is known that Asian people (Japanese) have a lower definition of obesity than the World Health Organization BMI cutoff point for obesity (≥30 kg/m^2),[13,14] which is controlled by genotypes. In a Japanese population, a strong association between visceral fat content and the metabolic syndrome has been reported, as seen even in subjects defined as nonobese by BMI but who were obese by CT.[34] In the present study the subjects who had the most significant weight gain and BP elevations also had a greater total body fat mass and waist-to-hip ratio plus higher plasma norepinephrine levels at entry, but BMI was not different between these entry groups. These findings suggest that abdominal obesity might be the link to heightened SNA, which is in part determined genetically by the abnormal β-adrenoceptor polymorphism. Alvarez et al[35,36] have reported that visceral obesity, but not subcutaneous obesity, is best associated with increased SNA. Grassi et al[37] have also found that central obesity is characterized by greater sympathetic activation and impaired baroreceptor sensitivity than peripherally obese or lean subjects.

In summary, these findings are from the first large cohort-based longitudinal study analyzing the effect of genetic variation in the β_2- and β_3-adrenoceptor genes over a fixed time period, showing their strong association with initiation of weight gain and BP elevation. SNA, as seen in plasma norepinephrine accompanying abdominal obesity, may be the major mediator of the β_2-adrenoceptor gene changes.

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β_2-Adrenoceptor Polymorphisms Relate to Insulin Resistance and Sympathetic Overactivity as Early Markers of Metabolic Disease in Nonobese, Normotensive Individuals

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Background: The genes responsible for insulin resistance are also candidate genes for insulin resistance-related diseases, such as obesity and hypertension. Functional polymorphisms in the β_2- and β_3-adrenergic receptors have been reported to be associated with diabetes, hypertension, and obesity. To clarify the relevance of the β-adrenergic receptor polymorphisms to insulin resistance, we studied their association with polymorphisms of β_2 (Arg16Gly, Gln27Glu) and β_3 (Trp64Arg) adrenoceptor genes.

Methods: We studied 155 young, nonobese Japanese men using the homeostasis model assessment of insulin resistance (HOMA-IR) to divide individuals into insulin-sensitive and insulin-resistant groups. Insulin resistance in the participants was defined as HOMA-IR equal to or greater than the average plus 1 SD of 3.1. There were 69 men who were insulin resistant and 86 men who were insulin sensitive. Body mass index (BMI), blood pressure (BP), plasma glucose, insulin, leptin, norepinephrine (NE) levels, and the polymorphisms of Arg16Gly and Gln27Glu of the β_2- and Trp64Arg of the β_3-adrenoceptor polymorphisms were measured in all participants.

Results: The insulin-resistant group had higher frequency of the Gly16 allele of Arg16Gly compared with the insulin-sensitive group, whereas the frequencies of genotypes or alleles of Gln27Glu and Trp64Arg were similar. The insulin-resistant group had a higher mean HOMA-IR, fasting insulin, NE, and total fat mass compared with levels in the insulin-sensitive group, but the BMI and leptin levels were similar. The subjects carrying the Gly16 allele of the β_2-adrenoceptor gene had a higher mean HOMA-IR, fasting insulin, NE, body fat mass, and BP than those without the Gly16 allele.

Conclusions: The Gly16 mutation of the β_2-adrenoceptor gene is associated with increased insulin resistance, adiposity, and BP accompanied by higher plasma NE levels early in the metabolic disease in developing obesity. These findings show an important role of β_2-adrenoceptor gene polymorphisms in the association of insulin resistance in hypertension and obesity. Am J Hypertens 2005;18:1009–1014 © 2005 American Journal of Hypertension, Ltd.

Key Words: Insulin resistance, sympathetic nerve activity, β_2- and β_3-adrenoceptor polymorphisms, blood pressure, and obesity.

Obesity and hypertension are associated with metabolic disturbances such as insulin resistance, hyperinsulinemia, and dyslipidemia.[1,2] One pathophysiologic significance of early insulin resistance is that insulin has mitogenic properties that can potentiate vascular smooth-muscle growth, promoting structural changes in blood vessels and possibly contributing to atherosclerosis. Thus, insulin resistance may be an important etiologic factor in the cardiovascular risk seen in the development of obesity and hypertension.[1–3]

One major risk is that human obesity and hypertension have well defined genetic determinants such polymorphisms in the β_2- and β_3-adrenergic receptor.[4–12] We have

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reported that insulin resistance and hyperinsulinemia are associated with heightened sympathetic nerve activity[13] and that heightened sympathetic nerve activity, as seen in elevated plasma NE, predicts insulin resistance, subsequent weight gain, and BP elevation.[14–16] In addition, normotensive and normal-weight individuals who have a positive family history of hypertension and obesity also have heightened sympathetic nerve activity.[17,18] These findings imply that sympathetic overactivity defined here as high plasma NE levels are associated with genetic determinants on the β-adrenergic receptor that may contribute to insulin resistance. The present study further examines the relationship between polymorphisms of the β-adrenergic receptors and progression in reduced insulin sensitivity in nonobese, normotensive Japanese men.

Methods

Subjects

A cohort of 1121 men working in Osaka, Japan, as part of their biannual medical evaluation were studied. Subjects were excluded who were >50 years of age, overweight (body mass index [BMI] 25 to 30 kg/m^2) or obese (BMI >30 kg/m^2), had diabetes mellitus (fasting glucose level>100 mg/dL), or hypertension (≥160/95 mm Hg). Additional exclusions were subjects who were taking medications for hypertension, hyperlipidemia, hyperuricemia, or other illness. After exclusion, 155 young men who were nonobese (BMI <25 kg/m^2) and nonhypertensive (BP <160/95 mm Hg) and who were not using any medications were recruited from the cohort. The subjects were subdivided into an insulin-sensitive group and an insulin-resistant group using the homeostasis model assessment of insulin resistance (HOMA-IR) and a cut-off limit of average + 1 SD (2.2 + 0.9) in participants. Because it is well known that recent alterations in plasma insulin, leptin, and NE levels are altered with weight changes, only those subjects who had steady body weight (weight had not changed significantly (<5%) over the past year) were enrolled in the present study.[15,16,19]

The protocol was approved by the Ethics Committee of Osaka University Graduate School of Medicine, Japan, and written informed consent was obtained from all of the subjects.

Measurements

After an overnight fast of 12 h, BMI, total body fat mass, BP, heart rate, and venous sampling for blood glucose, plasma norepinephrine (NE), insulin, leptin, and the extraction of genomic DNA from leukocytes were obtained after a 30-min rest period in the supine position. Lipids profiles (total cholesterol, triglyceride, HDL-cholesterol) and uric acid levels were also evaluated. Both BP and heart rate were measured three times in the supine position by an automated sphygmomanometer (TM-2713, A&D Co. Ltd., Tokyo, Japan), which had been standardized against a mercury sphygmomanometer. The percentage body fat mass was determined with impedance measurements (BF-102, Tanita, Japan), and total body fat mass (kg) was calculated according to the following formula: [percentage body fat mass (%)/100] × body weight (kg). Plasma NE was measured by high-performance liquid chromatography with a fluorometric method (intra-assay coefficient of variation [CV] = 2.1%; inter-assay CV = 3.6%; sensitivity = 0.06 to 120 nmol/L). Plasma immunoreactive insulin was measured by standard radioimmunoassay methods (insulin RIABEAD II, Dinabott; intra-assay CV = 1.9%; inter-assay CV = 2.2%; sensitivity = 0.75 to 300 μU/mL). Plasma leptin was measured by radioimmunoassay (human leptin RIA kit, Linco; intra-assay CV = 5.0%, interassay CV = 4.5%, and sensitivity = 0.03 to 6 nmol/L). The HOMA-IR was defined as the product of fasting plasma insulin (μU/mL) and glucose (mg/dL) divided by 405.[20]

Genotyping

Genotyping was performed by the TaqMan assay, as previously described.[21] Two polymorphisms (arginine/glycine substitution, Arg16Gly, and glutamine/glutamate substitution, Gln27Glu) of the β_2-adrenoceptors[6] and one polymorphism (tryptophan/arginine substitution, Trp64Arg) of the β_3-adrenergic receptor[11,12] were evaluated. For single-nucleotide polymorphisms of the β_2-adrenergic receptor gene, the probes and primers were as follows: for Arg16Gly, the probes were CGCATGGCTTCCATTGGGTGC and CGCATGGCTTCTATTGGGTGC, and the primers were GGAACGGCAGCGCCTTCT and CAGGACGATGAGAGACATGACGAT; for Gln27Glu, the probes were CTCGTCCCTTTCCTGCGTGACGT and CTCGTCCCTTTGCTGCGTGACGT, and the primers used in this assay were the same as those used for Arg16Gly. For the Trp64Arg single-nucleotide polymorphism in the β_3-adrenergic receptors, the probes were TCTCGGAGTCCAGGCGATGGCCA and CTCGGAGTCCGGGCGATGGCC, and the primers were GGAGGCAACCTGCTGGTCAT and CACGAACACGTTGGTCATGGT.

Statistical Analyses

Genotype frequencies and Hardy-Weinberg equilibrium were estimated with χ^2 test. Values are shown as mean ± SD. Differences among groups were examined by the paired or unpaired t test. Multiple regression linear analyses were applied to evaluate the relationship between HOMA-IR as a dependent variable and plasma NE, BMI, total body fat mass, and mean BP (systolic and diastolic BP) as independent variables. Values of $P < .05$ were considered significant.

Results

Prevalence of Insulin Resistance

A total of 69 subjects were insulin resistant and 86 subjects were insulin sensitive as defined by the HOMA-IR. The

insulin-resistant group had a significantly lower frequency of the Arg16/Arg16 genotype (χ^2= 12.38, P = .002) and a higher frequency of the Gly16 allele (χ^2= 5.53, P = .019) in analysis of the β_2-adrenoceptor gene compared with results in the insulin-sensitive group (Fig. 1). Frequencies of each genotype and allele of Gln27Glu and those of Trp64Arg were similar between the insulin-sensitive and insulin-resistant groups.

Profiles of Insulin-Resistant Subjects

The insulin-resistant group had higher HOMA-IR, fasting plasma insulin, NE, total body fat mass, uric acid, total cholesterol, triglyceride, and lower HDL-cholesterol levels, whereas BMI, BP levels, and leptin levels were similar in both groups (Table 1).

Profiles of the Subjects Carrying the Gly16 Allele of the β_2-Adrenoceptor

Insulin resistant subjects had a higher frequency of the Gly16 allele of the β_2-adrenoceptor gene, suggesting the Gly16 allele is related to insulin resistance. Thus, we compared the subjects with and without the Gly16 allele of the β_2-adrenoceptor gene regardless of the status of insulin sensitivity. The HOMA-IR, fasting plasmas insulin, NE, total body fat mass, serum uric acid levels, and systolic, diastolic, and mean BP levels were higher in the subjects with the Gly16 allele (the Arg16/Gly16 + Gly16/Gly16 genotype) compared with values in subjects without the Gly16 allele (the Arg16/Arg16 genotype) of the β_2-adrenoceptor gene (Table 2). When those subjects were subdivided by insulin sensitivity, only the insulin-resistant group with higher fasting plasma insulin ($P < .05$) and NE ($P < .05$) levels were found in the group with the Gly16 allele (Fig. 2).

Multiple Regression Linear Analyses

When HOMA-IR was used as a dependent variable, plasma NE (P = .012), total body fat mass (P = .016), and systolic (P = .034) and mean BP (P = .007) levels were significant determinant variables (R^2 = 0.379, F = 19.96, $P < .001$) in multiple regression linear analysis.

Discussion

To clarify the relationship of β-adrenoceptors polymorphisms, insulin resistance, and plasma NE levels as an index of the sympathetic nervous system activity, we studied profiles of hormones and relations of polymorphisms of β-adrenoceptor genes over time in healthy individuals. We found that the insulin-resistant subjects had higher frequencies of the Gly16 allele of the β_2-adrenoceptors, and that the subjects who carried the Gly16 allele had higher levels of fasting insulin (HOMA-IR), plasma NE, and uric acid. In addition, total body fat mass and BP levels were higher in the subjects with the Gly16 allele in nonobese, nonhypertensive men. These findings suggest 1) that insulin resistance could in part be determined by the genetic variant of the β_2-adrenoceptor gene, and 2) that the β_2-adrenoceptor polymorphism accompanying higher plasma NE levels could increase insulin resistance, adiposity (obesity), and BP elevation.

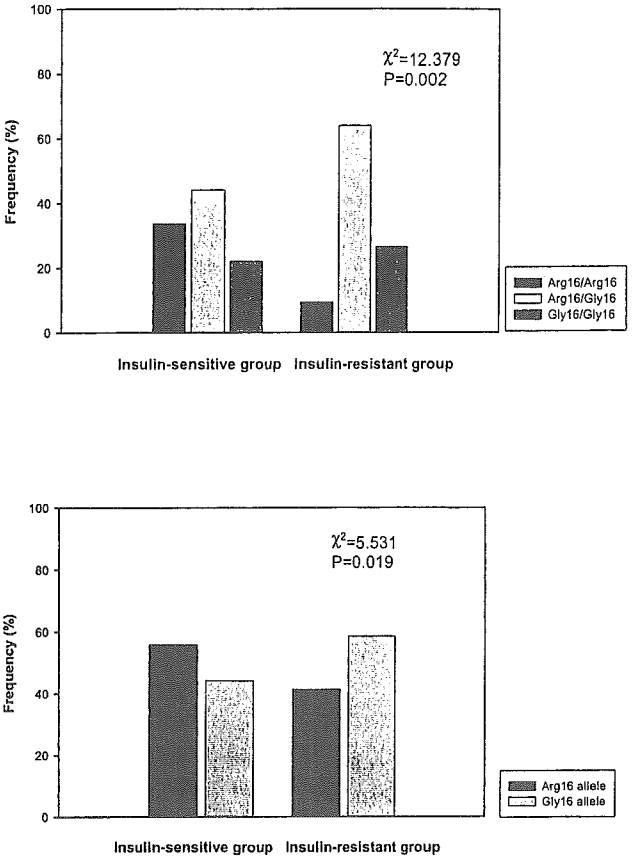

FIG. 1. Frequencies of the genotypes (**upper panel**) and the allele (**lower panel**) at Arg16Gly of the β_2-adrenoceptor gene.

In the present study, we used plasma NE levels as an index of sympathetic nerve activity. Tuck,[22] Grassi and Esler,[23] and Rahn et al[24] reported different results in sympathetic nervous system activity in hypertensive patients according to NE measurement methods, muscle sympathetic nerve activity using microneurography methods, plasma NE measurements, and regional spillover method.[25] Many investigators recommend the regional spillover method as a gold standard for sympathetic nerve activity, but these are difficult and invasive measurements. Plasma NE levels are much more practical for large populations and represent the result of several different processes such as secretion, clearance, and reuptake, especially in large population studies such as cross-sectional design studies[13,17] and in repeated measurements in longitudinal studies.[14–16]

β-Adrenoceptor Polymorphisms Versus Insulin Resistance

Significant evidence has been provided for a strong physiologic relationship between the β_2-adrenoceptor and β_3-adre-

Table 1. Comparisons of values between insulin-sensitive subjects and insulin-resistant subjects

Characteristic	Insulin-Sensitive Subjects	Insulin-Resistant Subjects
Number	86	69
Age (y)	37.0 ± 6.9	36.8 ± 7.8
Body mass index (kg/m^2)	21.6 ± 2.8	22.8 ± 2.8
Total body fat mass (kg)	14.4 ± 4.1	16.1 ± 3.9*
Waist-to-hip circumference ratio	0.90 ± 0.11	0.92 ± 0.13
Systolic blood pressure (mm Hg)	127 ± 12	129 ± 11
Diastolic blood pressure (mm Hg)	78 ± 11	79 ± 12
Mean blood pressure (mm Hg)	94 ± 10	96 ± 12
Heart rates (beats/min)	64 ± 3	65 ± 4
HOMA-IR	1.7 ± 0.9	4.2 ± 0.5†
Plasma insulin (μU/mL)	8.1 ± 2.7	17.1 ± 2.9†
Plasma norepinephrine (pmol/mL)	1.26 ± 0.29	1.74 ± 0.38*
Plasma leptin (ng/mL)	3.9 ± 2.0	4.1 ± 2.1
Blood glucose (mg/dL)	90.7 ± 5.8	93.5 ± 6.0
Total cholesterol (mg/dL)	200 ± 27	216 ± 22*
Triglyceride (mg/dL)	117 ± 35	173 ± 48‡
HDL-cholesterol (mg/dL)	58 ± 13	50 ± 12*
Uric acid (mg/dL)	5.3 ± 1.4	5.8 ± 1.2*

HOMA-IR = homeostasis model of insulin resistance.
* $P < .05$, † $P < .001$, ‡ $P < .01$ versus values in the insulin-sensitive subjects.

noceptor as seen in obesity,[6–9,11,26,27] hypertension,[6,10] and insulin resistance.[11,12] Among β_2- and β_3-adrenoceptor polymorphisms, amino acid substitutions, Arg16Gly and Gln27Glu of the β_2-adrenoceptor polymorphism, and Trp64Arg of the β_3-adrenoceptor polymorphism are also considered functionally important in understanding the genetic relationship among obesity, hypertension, and insulin resistance.

Gratze et al[28] found that young, normotensive, white male subjects homozygous for the Gly16 allele of the β_2-adrenoceptor gene had higher BP and lower peripheral vasodilation in response to the infusion of the β-blocker salbutamol. The β_2-adreoceptor is also expressed in pancreatic β-cells to modulate insulin secretion. Irakashi et al[29] suggested that the Arg16Gly variant of the β_2-adrenoceptor gene has an influence on insulin secretion. In the present study, the subjects with the Gly16 allele of the β_2-adreoceptor gene had higher plasma insulin and NE levels, suggesting that the Gly16 allele of the β_2-adrenoceptor gene is closely linked to insulin-resistant status

Table 2. Comparisons of values between subjects with and without Gly16 allele of the β_2- adrenoceptor gene

Characteristic	Subjects Without Gly16 Allele Arg16/Arg16	Subjects With Gly16 Allele (Arg16/Gly16 + Gly16/Gly16)
Number	45	110
Age (y)	36.2 ± 6.9	37.2 ± 6.5
Body mass index (kg/m^2)	21.6 ± 2.1	22.4 ± 2.5
Total body fat mass (kg)	14.6 ± 3.7	15.5 ± 3.9*
Waist to hip circumference ratio	0.90 ± 0.11	0.91 ± 0.13
Systolic blood pressure (mm Hg)	124 ± 12	129 ± 14*
Diastolic blood pressure (mm Hg)	76 ± 11	80 ± 12*
Mean blood pressure (mm Hg)	92 ± 12	96 ± 8*
Heart rates (beats/min)	63 ± 4	65 ± 3
HOMA-IR	2.5 ± 0.7	3.0 ± 0.5*
Plasma insulin (μU/mL)	10.2 ± 3.7	12.9 ± 2.2*
Plasma norepinephrine (pmol/mL)	1.28 ± 0.29	1.57 ± 0.38*
Plasma leptin (ng/mL)	3.8 ± 2.0	4.1 ± 2.1
Blood glucose (mg/dL)	91.0 ± 5.8	92.3 ± 6.0
Total cholesterol (mg/dL)	201 ± 30	210 ± 27
Triglyceride (mg/dL)	127 ± 43	148 ± 50
HDL-cholesterol (mg/dL)	57 ± 12	53 ± 13
Uric acid (mg/dL)	5.0 ± 1.4	5.7 ± 1.2*

Abbreviation as in Table 1.
* $P < .05$ versus values in the insulin-sensitive subjects.

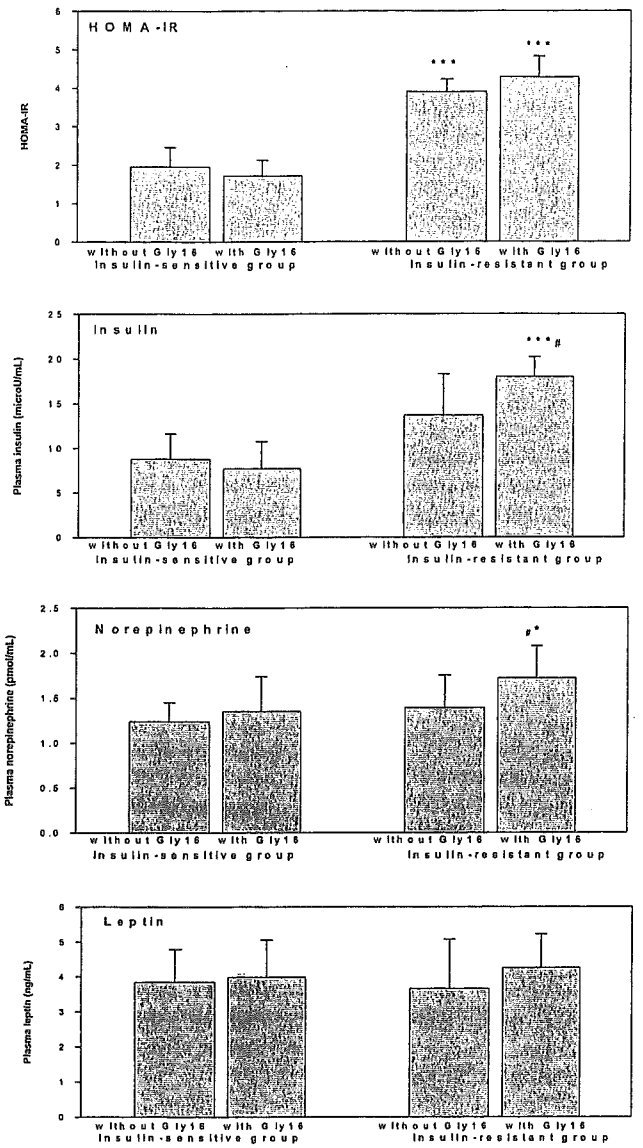

FIG. 2. The homeostasis model assessment of insulin resistance (HOMA-IR) (**top panel**), fasting plasma insulin levels (**second panel from top panel**), supine plasma norepinephrine levels (**second panel from bottom panel**), and plasma leptin levels (**bottom panel**) in the insulin-sensitive group and the insulin-resistant group according to the Gly16 allele of the β_2-adrenoceptor gene. $^{*}P < .05$, $^{***}P < .001$ versus values in the insulin-sensitive subjects. $^{\#}P < .05$ versus values in the subjects without Gly16 allele (carrying Arg16/Arg16 genotype) of the β_2-adrenoceptor gene.

associated with heightened sympathetic nerve activity shown as higher plasma NE levels and BP elevation. Thus, the Gly16 allele could lead to heightened sympathetic nerve activity, insulin resistance, and higher BP and adiposity and could predict these developments in nonobese, nonhypertensive individuals.

The Glu27/Glu27 genotype of the β_2-adrenoceptor gene has a well established association with obesity.[7] Subjects with Glu27 homozygotes have excess body fat and increased fat cell size compared with Gln homozygotes in a white population and also have abdominal obesity and insulin resistance.[26] We did not observe the association of the polymorphism at Gln27Glu of the β_2-adrenoceptor gene with insulin resistance, perhaps because of the very low frequency of the Glu27 allele. In a healthy Japanese population, distribution of the Glu27 allele of the β_2-adrenoceptor gene is different from that in individuals of non-Asian white ethnicity, as previously reported,[30] and the frequency of the Glu27 allele of the β_2-adrenoceptor gene is much lower.

Insulin Resistance Versus Sympathetic Overactivity

The group with the Gly16 allele of the β_2-adrenoceptor gene had a higher total body fat mass and BP levels, and our results in multiple regression analyses showed close relationships between HOMA-IR, plasma NE, total body fat mass, and mean BP. These findings demonstrate that the Gly16 allele that accompanies insulin resistance and heightened sympathetic nerve activity is associated with relatively greater adiposity and BP elevation. In addition, we have previously shown that insulin resistance is strongly related to heightened sympathetic nerve activity, BP elevation, and increased adiposity.[13–15,19] The present study was examined in a cross-sectional design. Hence, we could not discern the relations between genotype, BP elevation, and weight gain; however, we have reported in longitudinal studies that higher levels of plasma NE as a phenotype marker of sympathetic nerve activity predicts subsequent BP elevation and weight gain.[14–16] Taken together, our findings suggest the proposal that the adrenergic receptor defects lead to sympathetic nervous system overactivity that might play a role in the development of insulin resistance, hypertension, and obesity. In conclusion, a polymorphism at Arg16Gly of the β_2-adrenoceptor gene could be linked to insulin resistance and sympathetic nerve overactivity, as in this population of nonobese, nonhypertensive Japanese men.

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Rebound Weight Gain as Associated With High Plasma Norepinephrine Levels That Are Mediated Through Polymorphisms in the β2-Adrenoceptor

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Background: A successful weight loss program is essential treatment for obesity-related diseases, but it is well known that the majority of individuals do not succeed in weight loss maintenance. The present study evaluates hormonal mechanisms and the relationship of β2-adrenoceptor polymorphisms involved in individuals who regain weight after initially successful weight loss.

Methods: Overweight Japanese men (n = 154) were enrolled in a 24-month weight loss program. Body mass index (BMI), total body fat mass, plasma norepinephrine (NE) and leptin levels, and β2-adrenoceptor polymorphisms (Arg16Gly, Gln27Glu) were measured every 6 months for the 24-month period. Maintenance of weight loss was defined as significant weight loss (≥10% reduction) from entry weight at 6 months and maintenance of the weight loss for an additional 18 months. Rebound weight gain was defined as significant weight loss at 6 months but subsequent regain of body weight during the next 18 months.

Results: The results showed that 37 subjects maintained weight loss during 24 months, whereas 36 subjects had rebound weight gain. The BMI at entry and calorie intake and physical activity at each period were similar between the two groups. Subjects who maintained weight loss had at entry a significantly lower fat mass and plasma NE levels compared to those with rebound weight gain. Body fat mass, NE, and leptin levels at entry predicted the degree of change in body weight during the 24-month study period. Subjects with rebound weight gain had a significantly higher frequency of the Gly16 allele for the β2-adrenoceptor polymorphism compared to subjects who had a 24-month maintenance of weight loss. Subjects carrying the Gly16 allele also had significantly higher plasma NE, leptin, and body fat mass levels and a greater waist-to-hip ratio both at entry and throughout the study.

Conclusions: A high initial degree of body fat mass and high plasma NE levels as determined by the Gly16 allele for the β2-adrenoceptor polymorphisms predict those individuals who will have rebound weight gain after their initial successful weight loss. Am J Hypertens 2005;18:1508–1516 © 2005 American Journal of Hypertension, Ltd.

Key Words: Rebound weight gain, weight loss resistance, sympathetic nerve activity, leptin, obesity, β2-adrenoceptor polymorphisms.

Weight loss and maintenance of weight loss are the most effective nonpharmacologic treatments for correction of cardiovascular and metabolic risk factors in obese patients.[1–7] However, few obese people succeed in sustained weight loss, and long-term results of weight loss programs are disappointing with a substantial proportion of people regaining most of the weight initially lost.

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There is strong evidence suggesting that human obesity has both genetic and environmental determinants.[8,9] Investigations have reported associations of polymorphisms of the β2- and β3-adrenoceptors in obesity,[10–15] and regulation of thermogenesis is mainly attributed to β2- and β3-adrenergic receptor activity. Increased energy expenditure and increased resting metabolic rate are predictive

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of weight loss, and the sympathetic nervous system plays a key role in regulating energy balance through stimulation of thermogenesis. Effects on rates of thermogenesis are also influenced by genetic factors. Few studies have taken into account success in maintenance of weight loss, resistance to weight loss, and rebound weight gain as part of hormonal changes associated with changes in body weight or the polymorphisms of the β-adrenoceptor genes that occur with weight change. We examined weight loss in relation to changes in body fat mass, plasma norepinephrine (NE), leptin, and insulin. In addition, we compared polymorphisms of β2- and β3-adrenoceptor genes in subjects who maintained weight loss during 24 months to those who regain body weight (rebound weight) in a protocol of a defined, constant dietary intake and exercise program.

Methods

Subjects

The weight loss program enrolled 154 overweight (25 kg/m^2 ≤body mass index [BMI] <30 kg/m^2) men, consisting of 89 overweight normotensive men (blood pressure [BP] <140/90 mm Hg) and 65 overweight, untreated mildly hypertensive men (140/90 mm Hg ≤BP <160/95 mm Hg). None of the subjects had diabetes (fasting blood glucose level >100 mg/dL) or other illness including psychological or emotional problems.[16] No subject was taking antihypertensive agents or other medications. Furthermore, no subject had any symptoms of obstructive sleep apnea (ie, breathing pauses every night or almost every night) or extremely loud habitual snoring or sleepiness during the daytime.[17,18] Only subjects whose body weight had not changed for at least the past 2 years (weight change <5%) provided in their biannual medical evaluation records were enrolled in the present study.[4,19] The subjects enrolled in this weight loss program were emotionally stable,[16] and had a similar socioeconomic status. The protocol was approved by the Ethics Committee of Osaka University Graduate School of Medicine, Japan, and written informed consent was obtained from all the subjects.

Study Design

The weight loss program consisted of a low caloric diet (1600 kcal/d, 55% of calories from carbohydrate, 30% from protein, and 15% from fat) and a low sodium diet (7g NaCl per day) and aerobic exercise of more than 1 h daily (eg, walking, jogging, or gym exercise). The subjects attended a 1-h private teaching and counseling session each week for 4 weeks, followed by biweekly 1-h sessions for 23 additional months. All sessions were led by experts in nutrition and exercise counseling. Calorie intake was calculated based on the subjects meal diary, which was assisted by nutritionists. The physical activity was quantified and recorded by the use of step-counters used on a daily basis. Diet and exercise compliance were monitored according to the subjects' own records every 2 weeks and were recorded at private counseling sessions. Compliance to diet and exercise was considered excellent and consistent based on those records.

Methods

After an overnight fast of 12 h and 30 min rest in the supine position, height, body weight, BMI, percentage total body fat mass, BP, heart rate, and venous blood sampling for measurements of blood glucose, plasma NE, leptin, insulin, and the extraction of genomic DNA from leukocytes were obtained. Samples were taken at entry and at 6, 12, and 24 months during the study. The BP and heart rate were measured more than three times in the supine position by an automated sphygmomanometer (TM-2713, A&D, Tokyo, Japan) using an adjusted cuff size based on arm circumference. Recorded BP levels and heart rates were averaged. The percentage body fat mass was determined by impedance measurements (BF-102, Tanita, Tokyo, Japan). Total body fat mass (in kilograms) was calculated according to the following formula: [percentage body fat mass (%)/100] × body weight (kg). Plasma NE was measured after separation by high-performance liquid chromatography using the fluorometric method as previously described in detail,[19] and plasma immunoreactive insulin was measured by a standard radioimmunoassay method as described in detail (insulin RIABEAD II, Dinabott, Tokyo, Japan).[19] Plasma leptin was measured by radioimmunoassay[19] (human leptin RIA kit, Linco, St. Charles, MO, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was defined as the product of fasting plasma insulin (in microunits per milliliter) and glucose (in milligrams per deciliter) divided by 405.[20]

Genotyping

Genotyping was performed by the TaqMan assay, as previously detailed (Applied Biosystems, Foster City, CA, USA).[21] Two polymorphisms (Arg16Gly, Gln27Glu) of the β2-adrenoceptors[13,14] and one polymorphism (Trp64Arg) of the β3-adrenoceptor[22] were evaluated. For single-nucleotide polymorphisms (SNPs) in the β2-adrenoceptor genes, the probes and primers were as follows: for Arg16Gly, the probes were CGCATGGCTTCCATTGGGTGC and CGCATGGCTTCTATTGGGTGC, and the primers were GGAACGGCAGCGCCTTCT and CAGGACGATGAGAGACATGACGAT; and for Gln27Glu, the probes were CTCGTCCCTTTCCTGCGTGACGT and CTCGTCCCTTTGCTGCGTGACGT (the primers used in this assay were the same as those used for Arg16Gly). For the Trp64Arg SNP in the β3- adrenoceptor, the probes were TCTCGGAGTCCAGGCGATGGCCA and CTCGGAGTCCGGGCGATGGCC, and the primers were GGAGGCAACCTGCTGGTCAT and CACGAACACGTTGGTCATGGT.