The I/D Polymorphism of Angiotensin-Converting Enzyme Gene but not the Angiotensinogen Gene is Associated with Insulin Response to Oral Glucose in Japanese (44343)

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Abstract. **Polymorphisms of angiotensin-converting enzyme (ACE) and angiotensinogen (AGT) genes have been reported to be associated with myocardial infarction and coronary artery disease, both of which are closely related to atherosclerosis and insulin resistance. In this study, we investigated the association between ACE and AGT genotypes and insulin sensitivity in a sample of 142 nondiabetic and 64 noninsulin-dependent diabetes mellitus (NIDDM) Japanese subjects, aged 62.7 ± 9.5 years. The insulin response to the 75-g oral glucose tolerance test (OGTT) was significantly lower in subjects with the ACE D/D genotype compared to those with the I allele (I/D and I/I genotypes) in both nondiabetic ($P < 0.05$) and NIDDM subjects ($P < 0.005$). These homozygous D/D subjects also had lower insulin area under the curve of plasma insulin concentrations during OGTT compared to those with the I allele in nondiabetic ($P < 0.05$) and NIDDM subjects ($P < 0.01$). However, there was no significant association between AGT genotypes and either insulin response or insulin area under the curve during OGTT, in either nondiabetic or NIDDM subjects. From a viewpoint that insulin response to oral glucose is significantly correlated with insulin sensitivity, these results suggest that polymorphic variations at the ACE gene, but not the AGT gene, may be involved in the genetic regulation of insulin sensitivity in both nondiabetic and NIDDM Japanese subjects.** [P.S.E.B.M. 1999, Vol 220]

Angiotensin-converting enzyme (ACE) and angiotensinogen (AGT) genes are the major components of the renin angiotensin system. The ACE gene is located on human chromosome 17q23 with an insertion/deletion (I/D) polymorphism resulting from presence/absence of a 287 base-pair (bp) fragment in the 16th intron. The AGT gene was mapped on chromosome 1q42–43 and has a polymorphism at codon 235 in exon 2, composing the thymin-cytosine transition at nucleotide 701 that results in methionine-to-threonine substitution at amino acid 235 (M235T). The other polymorphism is located at codon 174 in the same exon as AGT codon 235. This molecular variant of AGT also constitutes the cytosine-thymine transition at nucleotide 521 that results in a threonine-to-methionine substitution at amino acid 174 (T174M).

Polymorphic variations at the ACE gene are reported to be associated with serum ACE concentration (1, 2), myocardial infarction (MI) (3–5), and coronary artery disease (CAD) (2, 4, 6–8); however, the association remains controversial (9). Polymorphic variations at the AGT codon 235 are documented to be associated with plasma concentration of AGT (10, 11), MI (5), CAD (8, 12), and essential hypertension (10, 13, 14); however, other studies have not been able to confirm the association (15, 16). Furthermore, the AGT codon 174 genotypes are shown to be associated

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This study was supported by the research fund of the Department of Internal Medicine I, School of Medicine, Oita Medical University.

Received May 1, 1998. [P.S.E.B.M. 1999, Vol 220]
Accepted September 14, 1998.

0037-9727/99/2201-0046$10.50/0

with essential hypertension (10, 15), CAD in low-risk subjects (8), and body fat distribution in men (17), but not in all studies (18, 19).

The role of insulin resistance in the pathogenesis and clinical course of the above diseases (e.g., essential hypertension and CAD) has been well established. Recent reports have implicated the association between ACE genotypes and insulin sensitivity. Subjects with ACE D/D genotype have a higher insulin sensitivity in nondiabetic Caucasian (20) and African-American (21) subjects, but not in noninuslin-dependent diabetes mellitus (NIDDM) Caucasian subjects (20). However, Panahloo *et al.,* (22) reported similar association in NIDDM but not in nondiabetic Caucasians. The frequency of the D/D genotype is lower in Japanese (5, 8) than in Caucasians (3, 4, 20, 22) and African-Americans (21). In the present study, we investigated the role of the ACE gene on insulin response during an oral glucose tolerance test (OGTT) in a Japanese population, whose insulin response is accepted to be generally low compared with that of Caucasians (23). We further tested the hypothesis that the AGT gene is also associated with insulin sensitivity.

Materials and Methods

Subjects and Study Design. In the present study, 206 (148 males and 58 females) consecutive Japanese subjects, aged 62.7 ± 9.5 years (mean ± SD), who were admitted to our department for diagnosis or treatment, including patients with angina pectoris, valvular diseases, arrhythmia, cardiomyopathy, and essential hypertension, participated. Written informed consent was obtained from all of the subjects. After an overnight fast, blood samples were obtained for measurement of plasma glucose and insulin. Subjects were given a 75-g oral glucose challenge, and blood was sampled again 30, 60, 90, and 120 min later for the measurement of plasma glucose and insulin. The area under the curve of plasma insulin concentrations from 0 to 120 min was calculated for each subject. Plasma glucose was assayed with the glucose oxidase method. Plasma insulin was assayed using a double-antibody radioimmunoassay. Results of the 75-g OGTT were assessed according to the WHO criteria (24), by which the subjects were divided into nondiabetic ($n = 142$) and NIDDM ($n = 64$). The nondiabetic group was composed of 66 subjects with normal glucose tolerance and 76 subjects with impaired glucose tolerance. Out of the 64 NIDDM patients, 18 had been previously diagnosed as NIDDM (on dietary therapy alone). None of the NIDDM subjects had been treated with insulin or oral hypoglycemic agents previously. Other factors known to potentially influence OGTT, such as age, sex, body mass index (BMI, kg/m^2), and hypertension, were concomitantly assessed. Hypertension was defined according to the WHO criteria (i.e., systolic blood pressure (BP) $\geqslant$ 160 mmHg and/or diastolic BP $\geqslant$ 95 mmHg) (25). The hypertensives included those with current hypertension and those with antihypertensive drugs together with a previous history of hypertension.

Genotype Determination. Genomic DNA was extracted from white blood cells by a standard method as previously described (26). For the typing of the insertion (I) and deletion (D) alleles of the ACE gene, genomic DNA was amplified by the polymerase chain reaction (PCR) reported by Chiu *et al.* (21). Since overestimation of the D allele could result from failure to amplify the I allele in the heterozygous (I/D) state, this mistyping was avoided in the present study by adding 5% dimethylsulfoxide (DMSO) and insertion-specific primer to the PCR reaction mixture. The sequences of the upstream, downstream, and insertion-specific primers were 5′-CTG GAG ACC ACT CCC ATC CTT TCT-3′, 5′-GAT GTG GCC ATC ACA TTC GTC AGA T-3′, and 5′-TCG AGA CCA TCC CGG CTA AAA C-3′, respectively. Reactions were performed in a final volume of 20 μl containing 100 ng template DNA, 20 pmol of each primer (upstream, downstream, and insertion-specific primers), 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 0.1 mg/ml gelatin, 0.5 mmol/l of each dNTP, 5% DMSO, and 0.5 unit (U) of Taq DNA polymerase (TAKARA). Amplification was carried out in a Thermal sequencer TSR-300 (IWAKI GLASS Co., Ltd., Funabashi, Japan). After entailment 3 min of denaturation at 94°C, the cycling conditions were as follows; 30 cycles with steps of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, and a final extension time of 10 min. PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide for allele identification, where a 192-bp fragment in the absence of the insertion (D), and a 479 bp and/or a 277 bp fragments in the presence of the insertion (I) (21).

For the AGT codon 174 typing, DNA was amplified by PCR; conditions were modified from the protocol of Caulfied *et al.* (18). Upstream and downstream primer sequences were 5′-GAT GCG CAC AAG GTC CTG-3′ and 5′-AGG GTG CTG TCC ACA CTG GAC CC-3′, respectively. PCR conditions were the same as for detection of the ACE genotype. The expected size of the PCR product of 303 bp was verified by electrophoresis in 1.5% agarose gel containing ethidium bromide. The 2-μl unpurified PCR product was digested with 0.7 U Nco I restriction endonuclease (Toyobo Co., Ltd., Osaka, Japan) at 37°C for at least 5 hr. Digested fragments were separated by electrophoresis in 1.5% agarose gel containing ethidium bromide. The homozygous threonine allele of the AGT codon 174 gene (T174) appears as a nondigested single 303-bp band and the M174 genotype as digested 211- and 92-bp bands.

M235 and T235 alleles of the AGT gene were detected by the method of Morise *et al.* (15) with some modifications. Upstream and downstream primer sequences were 5′-TGA CAG GAT GGA AGA CTG GCT GCT CCC TGC-3′ and 5′-AGC AGA GAG GTT TGC CTT ACC TTG-3′, respectively. PCR conditions were the same as above except that the annealing temperature was 68°C. The expected size of the PCR product was 104 bp. The 1-μl unpurified PCR product was digested with 1 U Msp I restriction endonuclease (Toyobo Co., Ltd.) at 37°C for at least 5 hr. Digested fragments were separated by electrophoresis in 3% NuSieve (FMC Bio Products-Rockland, MD, USA) agarose gel containing ethidium bromide. The homozygous methionine allele of the AGT codon 235 gene (M235) appears as a nondigested single 104-bp band and the T235 genotype as digested 73- and 31-bp bands.

Statistical Analysis. Previous studies suggest that the effect of ACE genotypes on insulin resistance (20–22) and ischemic heart disease (including MI and CAD) (2–5, 6–8) is not linear or codominant. In these studies, subjects with the D/D genotype have higher serum ACE concentration (1–2) and experience higher risk of MI (3–5) and CAD (2, 4, 6–8) compared to those with I/D and I/I genotypes. In this study, we hypothesized that the codominant effect is characterized by a higher insulin resistance among subjects with D/D genotype relative to those with I/D and I/I genotypes in Japanese. Therefore, the analysis was conducted to test this hypothesis by combining data in the I/D and I/I genotypes and comparing them to those of the D/D genotype. StatView J-4.5 was used for the statistical analysis. Because the distribution of plasma glucose and insulin concentrations during OGTT was skewed, the logarithmic transformation (base e logarithm) of each value was applied to normalize the data before performing statistical analysis. The chi-square test was carried out for comparisons of genotype frequencies. Variables of plasma glucose and insulin concentrations during OGTT and other clinical background profiles were analyzed by two-way analysis of variance (ANOVA) or unpaired *t* tests. A P value < 0.05 was considered to be statistically significant.

Results

Among the 206 subjects, the distribution of ACE genotypes was as follows: D/D 14.6%, I/D 43.7%, and I/I 41.7%; making the allelic frequency (D) of 0.364. There was no significant difference in gene frequency between nondiabetic and NIDDM subjects ($P = 0.91$) (Table I). Distribution of ACE genotypes followed closely the Hardy-Weinberg equilibrium law. Among nondiabetic subjects, no significant difference in genotype between subjects with normal glucose tolerance and those with impaired glucose tolerance was observed (data not shown). Also, there was no significant difference in genotype frequencies of the AGT codon 235 (M235, M235T and T235) and the AGT codon 174 (T174, T174M and M174) genotypes between nondiabetic and NIDDM subjects (data not shown).

Table I. Distribution of Genotypes and Allele Frequencies of ACE Gene

	Nondiabetic	NIDDM	Total
Genotype frequency, *n* (%)			
D/D	21 (14.8)	9 (14.1)	30 (14.6)
I/D	65 (45.8)	25 (39.0)	90 (43.7)
I/I	56 (39.4)	30 (46.9)	86 (41.7)
Total	142	64	206
Allelic frequency			
D	0.377	0.336	0.364
Standard error	0.029	0.042	0.024

Note. ACE: angiotensin-converting enzyme; NIDDM: noninsulin-dependent diabetes mellitus. There was no significant difference between the nondiabetic and NIDDM groups in terms of genotype frequency ($P = 0.91$). The distribution of genotypes in both groups follows closely the Hardy-Weinberg equilibrium law.

The nondiabetic and NIDDM groups were comparable in terms of the clinical profiles (Table II), according to ACE genotypes. However, subjects with the ACE D/D genotype had a higher level of plasma insulin concentrations throughout OGTT than those with I/D and I/I genotypes in both nondiabetic and NIDDM groups ($P < 0.05$ and $P < 0.005$, respectively; Table III). The area under the curve of plasma insulin concentrations from 0 to 120 min among the D/D subjects was 29% lower than those with the I allele in the nondiabetic group ($P < 0.05$); the difference was more pronounced in the NIDDM group (47%, $P < 0.01$; Table III). Nevertheless, there was no significant difference between subjects with the ACE D/D genotype and those with the I allele in terms of plasma glucose levels in either NIDDM or nondiabetic subjects. When hypertension was considered in the ANOVA model as a factor, these results remained unchanged. Also, there was no interaction effect between hypertension and diabetic status in plasma insulin concentrations and glucose level.

The glucose and insulin response to oral glucose as well as insulin area under the curve of plasma insulin were not significantly different among the genotypes of AGT codon 235 or AGT codon 174 in either nondiabetic or NIDDM groups (data not shown).

Discussion

The relationship between ACE genotype and insulin sensitivity in Japanese subjects has not been reported so far. Results of the present study suggest that subjects who are homozygous for the ACE D allele (e.g., D/D genotype) may be more insulin-sensitive than their counterparts with the I allele (I/D and I/I genotypes) in both nondiabetic and NIDDM Japanese subjects. On the other hand, such a relationship was not evident for AGT genotypes in this population.

It is conceivable that factors other than insulin sensitivity may account for the insulin response to glucose (27–29). Nevertheless, it has been shown that insulin response to oral glucose is significantly correlated with insulin sensitivity in normal subjects since an insulin response could account for approximately one-third of the total variance in insulin action (30).

The present results are consistent with those reported in Caucasian and African-American subjects (20–22). For instance, among nondiabetic Caucasian subjects with the homozygous D/D genotype, a lower plasma insulin response to oral glucose and a higher insulin sensitivity compared to

Table II. Clinical Characteristics of Study Subjects According to ACE Genotype

	Nondiabetic		NIDDM	
	D/D	I/D + I/I	D/D	I/D + I/I
Number of subjects	21	121	9	55
Sex (male/female	16/5	83/38	7/2	42/13
Age (year)	62.9 ± 10.6	63.0 ± 9.6	60.5 ± 9.7	61.3 ± 9.5
BMI (kg/m^2)	23.9 ± 7.8	23.7 ± 2.5	25.9 ± 7.2	25.1 ± 3.1
SBP (mmHg)	129.9 ± 12.0	130.9 ± 17.8	136.8 ± 20.2	135.1 ± 19.6
DBP (mmHg)	74.4 ± 8.0	73.7 ± 12.8	80.0 ± 15.7	76.9 ± 12.6
Hypertension (+)/(−)	7/14	45/76	5/4	30/25

Note. Values represent mean ± SD; ACE: angiotensin-converting enzyme; NIDDM: noninsulin-dependent diabetes mellitus; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure. There was no significant difference between the two groups in any parameters.

Table III. Results of Oral Glucose Tolerance Test (OGTT) by ACE Genotype

	Nondiabetic		NIDDM	
	D/D	I/D + I/I	D/D	I/D + I/I
Ln plasma (Ln mmol/l)				
Fasting	1.60 ± 0.06	1.61 ± 0.13	1.73 ± 0.11	1.73 ± 0.17
30 min	2.13 ± 0.18	2.22 ± 0.17	2.33 ± 0.17	2.37 ± 0.35
60 min	2.17 ± 0.29	2.25 ± 0.27	2.50 ± 0.19	2.55 ± 0.20
90 min	2.10 ± 0.32	2.18 ± 0.28	2.46 ± 0.33	2.60 ± 0.44
120 min	2.00 ± 0.26	2.04 ± 0.27	2.40 ± 0.45	2.50 ± 0.32
Ln plasma insulin (Ln μ U/ml)[a]				
Fasting	1.01 ± 0.80	1.33 ± 0.75	0.96 ± 0.62	1.54 ± 0.59
30 min	3.39 ± 0.67	3.58 ± 0.94	2.57 ± 0.83	3.20 ± 0.84
60 min	3.42 ± 0.72	3.85 ± 0.87	3.10 ± 0.74	3.63 ± 0.88
90 min	3.36 ± 0.83	3.81 ± 0.89	3.00 ± 0.70	3.82 ± 0.80
120 min	3.35 ± 0.84	3.77 ± 0.83	3.16 ± 0.70	3.99 ± 0.79
Ln insulin area under the curve[b]	4.08 ± 0.54	4.42 ± 0.73	3.67 ± 0.39	4.30 ± 0.68

Note. Values represent mean ± SD; ACE: angiotensin-converting enzyme; NIDDM: noninsulin-dependent diabetes mellitus; Ln: base e logarithm.

[a] $P < 0.05$ and $P < 0.005$ (for nondiabetic and NIDDM, respectively) comparing D/D and I/D + I/I throughout OGTT using two-way ANOVA.

[b] $P < 0.05$ and $P < 0.01$ (for nondiabetic and NIDDM, respectively) comparing D/D and I/D + I/I using the unpaired Student's *t* test.

those with the heterozygous I/D genotype as assessed by steady-state plasma glucose (SSPG) was observed (20). In the same study, the authors also reported a similar but insignificant trend in NIDDM subjects. Another study, by using the homeostatic model assessment (HOMA), showed that D/D subjects are associated with higher insulin sensitivity in NIDDM but not in nondiabetic Caucasians (22). They suggested an interaction between diabetes and the renin-angiotensin system in insulin sensitivity. Furthermore, Chiu *et al.* (21) demonstrated that the D/D genotype is associated with not only lower insulin response to oral glucose and lower insulin area under the curve but also higher insulin sensitivity by the euglycemic clamp method in glucose tolerant and normotensive subjects. However, the sample size was small in their study, which might come from the limitations associated with the glucose clamp technique. Inconsistent results between the studies may also be due to sampling fluctuation and different methods of measurement of insulin sensitivity and genotyping. Previous studies on Caucasian subjects used old methods for genotyping of the ACE gene (31, 32). We employed the method reported by Chiu *et al.* (21) to avoid the mistyping. On the other hand, a gene-environmental interaction effect, which is likely present in different populations, cannot be ruled out.

Numerous reports have demonstrated the association between ACE genotypes and ischemic heart disease, including MI and CAD (2–5, 6–8), both of which are known to be related to insulin resistance. We initially speculated that the ACE D/D genotype may be associated with lower insulin sensitivity; however, results of the present study and others were contrary. Indeed, some studies have shown that the D/D genotype may be associated with longevity. In those studies, the frequency of the D/D genotype was higher in a French population of centenarians (33) and in long-term survivors of insulin-dependent diabetes mellitus (34). If this ACE D/D genotype is associated with higher insulin sensitivity, then it may be more advantageous for longevity than other genotypes with the I allele.

How does the ACE D/D genotype relate to insulin sensitivity? The ACE D/D genotype was reported to be associated with a higher plasma ACE level than other genotypes (1, 2) that may result in higher plasma angiotensin II. Infusion of a low dose of angiotensin II increased insulin sensitivity (35–37). This phenomenon may be explained by the effects of angiotensin II on redistribution of blood flow from the visceral, particularly renal circulation toward skeletal muscle that may lead to an increase in glucose uptake and a decrease in renal flow producing reduced insulin excretion (36). Another potential mechanism is that angiotensin II has a direct effect to improve insulin sensitivity *via* different intracellular pathways that are involved in glucose utilization (38). For example, angiotensin II stimulates hepatic carbohydrate metabolism through a Ca^{2+}-requiring, cAMP-independent mechanism, which changes the phosphorylation state of key regulatory enzymes (39). Angiotensin II also has been reported to increase prostacyclin production (40). Moreover, angiotensin II mediates activation of protein kinase C *via* receptor-linked formation of diacylglycerol, which is related to insulin sensitivity of liver, muscle, and adipose tissues (41). Since ACE inhibitors reduce plasma angiotensin II concentration, it seems contradictory that they improve insulin sensitivity (42–45). However, this phenomenon can be explained by the fact that ACE inhibitors, such as captopril and enalapril, inhibit kinin degradation, and then the sustained kallikrein system including bradykinin increases peripheral insulin sensitivity and reduces hepatic glucose output (42).

Although subjects with the D/D genotype had higher insulin sensitivity than those with I/D and I/I genotypes, the allele D frequency in nondiabetic subjects was not significantly different from that in NIDDM subjects. It is highly possible that other genetic and environmental factors also contributed to the development of NIDDM. This view is also consistent with the currently acceptable hypothesis that NIDDM is a multifactorial trait.

In our Japanese sample, no significant association between the AGT codon 235 or the AGT codon 174 genotypes and insulin response to oral glucose was found (data not shown), although the AGT T235 genotype is reported to be associated with a higher plasma AGT level (10,11). One possible explanation for this observation is that elevated plasma angiotensinogen may be compensated by downregulation of plasma renin activity (46). This is in contrast with the relationship between the D/D genotype and elevated plasma ACE concentration, which directly upregulates the angiotensin II level. Although an increased level of angiotensin II may be a direct cause of higher insulin sensitivity as discussed above, other mechanisms such as linkage disequilibrium with another influential polymorphism in a nearby gene such as the growth hormone gene could be a possible link (47).

In conclusion, we report that polymorphic variations of the ACE gene, but not the AGT codon 235 and 174 gene, may be involved in the genetic regulation of plasma insulin response to oral glucose in both nondiabetic and NIDDM Japanese. Since the association of the ACE D/D genotype and insulin sensitivity has been reported in other ethnic populations (20–22), it may be a common phenomenon throughout different races. These results and others raise the question of why the incidence of the ACE D/D genotype is increased in MI and CAD irrespective of their merit in insulin sensitivity. The possible mechanisms underlying these phenomena need to be investigated further.

The authors thank the doctors in the Department of Internal Medicine I, Oita Medical University, for their cooperation; Prof. Davis S. Knight, Louisiana State University, and Prof. Tuan V. Nguyen, Wright State University School of Medicine, for review of the manuscript.

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