The Insertion Allele at the Angiotensin I–Converting Enzyme Gene Locus Is Associated With Insulin Resistance

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Plasma angiotensin I–converting enzyme (ACE) levels are genetically predetermined and are correlated with a deletion (D) insertion (I) polymorphism at the ACE gene locus. A subset of diabetic patients are noted to have elevated ACE levels. Treatment with ACE inhibitors has been shown to improve insulin sensitivity in both diabetic and nondiabetic subjects. We examined the relationship of D/I polymorphism and insulin sensitivity in 24 glucose-tolerant subjects by an oral glucose tolerance test (OGTT) and glucose clamps. Subjects with the I allele had higher insulin levels at 90 minutes (515 ± 69 *v* 250 ± 43 pmol/L, *P* = .008) and higher insulin area under the curve (56,200 ± 8,148 *v* 33,300 ± 8,114, *P* = .022) after glucose challenge compared with subjects without the I allele. During the euglycemic clamp, subjects with the I allele require less glucose infusion to maintain euglycemia than subjects without the I allele (5.343 ± 0.743 *v* 8.944 ± 1.272 mg/kg/min, *P* = .020). We conclude that the I allele is associated with insulin resistance in glucose-tolerant and normotensive African-Americans.

ANGIOTENSIN I–CONVERTING ENZYME (ACE) plays an important role in blood pressure regulation and electrolyte balance by hydrolyzing angiotensin I to angiotensin II, a potent vasopressor and aldosterone-stimulating peptide.[1,2] The enzyme is also able to inactivate bradykinin, a potent vasodilator.[1,2] Plasma ACE levels are stable in a given individual, but differ greatly among subjects.[3] It has been shown that in whites, 50% of the interindividual variability of plasma ACE concentrations is determined by a deletion (D) insertion (I) polymorphism in intron 16 of the ACE gene.[4] The mean plasma ACE level of D/D subjects is approximately twice that of I/I subjects, with I/D subjects having intermediate levels.[4,5] Various studies show that the D/I polymorphism at the ACE locus is associated with coronary artery disease in the general population[6] and in patients with non–insulin-dependent diabetes mellitus (NIDDM).[7]

Diabetes mellitus is one of the diseases associated with elevated plasma ACE levels,[8,9] and the majority of diabetic individuals with elevated plasma ACE levels have retinopathy, renal failure, or both.[10-13] Although the cause-effect relationship of elevated ACE levels in diabetic nephropathy and retinopathy is not established, it is clear that a subset of diabetic patients have elevated ACE levels. Furthermore, the D/I polymorphism at the ACE locus is associated with diabetic nephropathy.[14,15]

Although some reports have shown that ACE inhibitors have no effect on glycemic control,[16] a large body of evidence has shown that ACE inhibitors improve glycemic control in diabetic patients.[17] Furthermore, it has been demonstrated that insulin sensitivity improves after administration of ACE inhibitors in NIDDM patients,[18] nondiabetic hypertensive subjects,[19] and normal (normotensive and glucose-tolerant) subjects.[20]

In the present study, we examined the relationship between D/I polymorphism and insulin sensitivity in 24 glucose-tolerant and normotensive subjects by the standard oral glucose tolerance test (OGTT) and euglycemic clamp technique. We found that subjects with the I allele had higher insulin levels and lower glucose deposition rates compared with subjects without the I allele.

SUBJECTS AND METHODS

Subjects

To minimize confounding factors, we chose to focus our study on one ethnic group, African-Americans. Written informed consent was obtained from 25 African-Americans without a prior history of diabetes or hypertension. Subjects with a prior history of diabetes or hypertension were not recruited for this study, and only healthy volunteers without a prior history of diabetes or hypertension were invited to participate in the study. All subjects were invited for an initial screening study that included an OGTT, measurements of blood pressure, anthropometric measurements, and medical and family history, using the established protocol.[21] An OGTT was performed on every subject after overnight fasting for 12 hours. Fasting glucose and insulin values were averaged from the values obtained at −15, −10, and −5 minutes before administration of a 75-g glucose solution. Four additional blood samples were obtained at 30-minute intervals. Glucose was assayed immediately at bedside using the glucose oxidase method. Insulin was assayed using a double-antibody radioimmunoassay.[22] Blood pressure was measured three times, each 5 minutes apart, while the subject was resting in bed quietly.[21] The mean of the three measurements was used for analysis. Except for one subject with a blood pressure of 167/110 mm Hg, all were normotensive <140/90 mm Hg). The hypertensive subject was excluded from analysis, since hypertension is noted to be associated with insulin resistance. Blood pressure measurements used in the analysis were performed on the morning of the OGTT. Blood pressure measurements were performed again on the morning of the euglycemic clamp study. All subjects were normotensive during this second measurement performed immediately before the euglycemic clamp study. Subjects' clinical features are shown in Table 1.

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Table 1. Clinical Characteristics of the Subjects (N = 24)

Characteristic	Mean	SE	%
Age (yr)	32	2	
Gender (female, n)	16		67
Family history of diabetes (n)	11		46
Family history of hypertension (n)	12		50
Body mass index (kg/m^2)	28.8	1.1	
Waist to hip ratio	0.843	0.014	
Systolic blood pressure (mm Hg)	114	3	
Diastolic blood pressure (mm Hg)	71	2	
OGTT plasma glucose (mmol/L)			
Fasting	4.61	0.07	
30 min	6.67	0.27	
60 min	6.71	0.33	
90 min	6.11	0.25	
120 min	5.77	0.16	

Measurement of Insulin Sensitivity by Euglycemic Clamp

Based on the initial screening study, glucose-tolerant and normotensive volunteers were invited to participate in a euglycemic clamp study. After a 12-hour fast, euglycemic clamps were performed according to procedures described by DeFronzo et al.[23] In brief, a primed-continuous infusion of human insulin was administered at a rate of 40 mU/m^2/min. The infusion rate of a 20% glucose solution was adjusted every 5 minutes to maintain blood glucose at the preclamp (fasting) level for 2 hours. The glucose deposition rate was calculated from the mean glucose infusion rate required to maintain euglycemia during the last 30 minutes, based on body weight (per kilogram) and unit time (per minute).

DNA Analysis

Genomic DNA was extracted from peripheral lymphocytes as described previously.[24] Polymerase chain reaction (PCR) assay for ACE D/I polymorphism was performed in a final volume of 10 μL containing 10 pmol of each primer (primers 1, 2, and 3; Fig 1), 2 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris hydrochloride, pH 8.3, 0.001% gelatin, 5% dimethylsulfoxide (DMSO), 0.2 mmol/L of each dNTP, and 0.25 U Taq polymerase. The DNA was amplified by an initial denaturation at 94°C for 5 minutes, 30 cycles with denaturation at 94°C for 1 minute, annealing at 64°C for 1 minute, extension at 72°C for 1 minute, and then final extension at 72°C for 10 minutes. PCR products were resolved on 2.0% agarose gel. Genotyping was performed after initial screening studies and euglycemic clamps. The connection between genotypes and phenotypes was only established at the time of data analysis.

Statistical Analysis

Differences between groups with quantitative variables were evaluated by unpaired Student's *t* tests, and differences in proportions were evaluated by chi-square tests. Data that failed normality tests were evaluated by the Mann-Whitney test. Statistical significance was accepted at P less than .05.

RESULTS

It has been noted that at this locus, overestimation of the D allele is relatively common due to a failure to amplify the I allele in the heterozygous (D/I) state.[25] Without 5% DMSO or an insertion-specific internal primer, the published PCR assay for ACE polymorphism tends to mistype the D/I genotype and leads to an overestimation of the frequency of the D/D genotype.[6] Even in the presence of 5% DMSO, PCR with the original primer set (primers 1 and 2) failed to amplify the insertion allele (479 bp) in one of the D/I subjects (Fig 2). PCR with an insertion-specific internal primer (primer 3) is the only way to avoid mistyping at this locus. In the present study, we used both DMSO and an insertion-specific internal primer for PCR. Genotypes were confirmed in triplicate on different occasions.

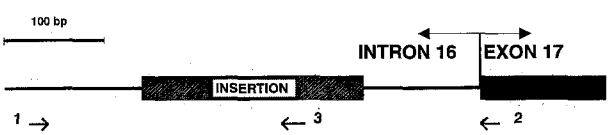
In 24 African-Americans, we found eight subjects with D/D, 11 with D/I, and five with I/I. The allelic frequencies (56% for D and 44% for I) were similar to the published results.[6] The observed genotypic frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium (32%, 49%, and 19%, respectively, or 7.6, 11.8, and 4.6, respectively), which supported the accuracy of the observed genotypic frequencies. Since only five subjects were identified with I/I, they were pooled with D/I subjects for data analyses. During the OGTT, none of the volunteers were noted to have a fasting or 2-hour glucose level greater than 7.7 mmol/L or an interval glucose level greater than 11.1 mmol/L (Table 1).

Clinical features of the two genotypic groups were compatible (Table 2). Plasma glucose levels of the two groups during the OGTT were similar. However, subjects with the I allele (D/I or I/I) had higher insulin levels at each time point compared with subjects without the I allele (D/D) (Table 3). This difference is significant, with a P value of .008 by Mann-Whitney test for plasma insulin level at 90 minutes. A similar result was observed when we compared the insulin area under the curve of the two genotypic groups (P = .022 by Mann-Whitney test).

As compared with the tightly maintained fasting plasma glucose, fasting plasma insulin varied drastically among different subjects. We also compared the stimulated insulin level (delta insulin) after glucose challenge at each time point by subtracting the fasting insulin level. The stimulated insulin level at 90 minutes in subjects with the I allele was much higher than in subjects without the I allele (P = .005 by Mann-Whitney test). Since a higher insulin level is an indirect indicator for insulin resistance, these observations suggest that subjects with the I allele are more insulin-resistant compared with those without the I allele. Glucose clamp studies were performed on 14 subjects with the I allele and six subjects without the I allele. The glucose deposition rate of subjects with the I allele was much lower than that of subjects without the I allele (5.343 ± 0.743 *v* 8.944 ± 1.272 mg/kg/min, P = .02; Fig 3). This result clearly indicates that subjects with the I allele are more insulin-resistant than subjects without the I allele.

DISCUSSION

It is important to avoid mistyping in a genetic study.[25,26] An inability[27] to replicate some association studies' results[28,29] at this locus is well recognized as being due to an overestimation of the D/D genotype. For those studies, the observed genotypic



Primer 1: CTGGAGACCACTCCCATCCTTTCT

Primer 2: TCGAGACCATCCCGGCTAAAAC

Primer 3: GATGTGGCCATCACATTCGTCAGAT

Fig 1. Schematic diagram of PCR design and primers.

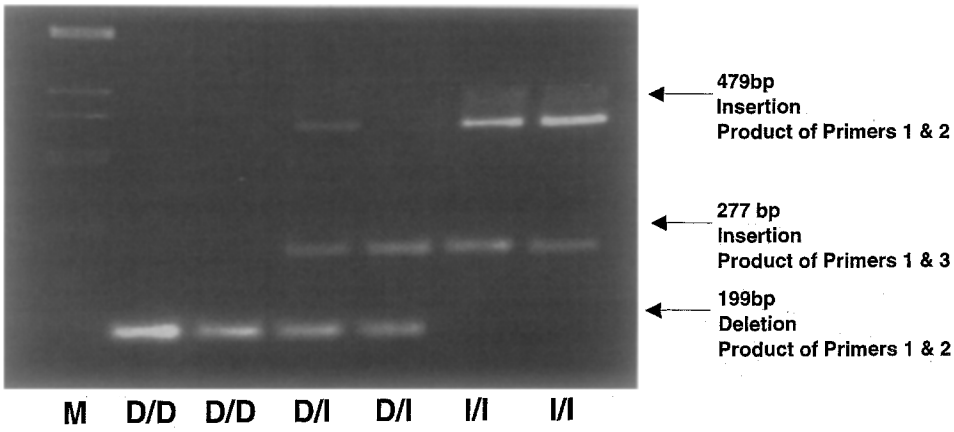


Fig 2. Ethidium bromide–stained 2% agarose gel of PCR products.

frequency failed to comply with the Hardy-Weinberg equilibrium. Deviation (less than predicted) from Hardy-Weinberg equilibrium is expected if the genotype or allele is unfavorable for survival (increased mortality). Therefore, an allele or genotype with a higher observed frequency than the predicted one is not likely to be associated with a disease status, unless the disease itself prolongs life. To avoid the same pitfall, we used both DMSO and an insertion-specific primer in our PCR assay with repeated testing. Even with the addition of DMSO, mistyping can still occur (Fig 2). Thus, genotyping using PCR with an insertion-specific internal primer (plus repeated testing) is the only safeguard against mistyping at this locus. The alternative method is to use Southern blotting of genomic DNA that is digested by a restriction enzyme.[15] However, the latter requires a relatively large amount of genomic DNA and is time-consuming and labor-intensive.

Recently, by modeling from fasting insulin and glucose levels (homeostatic model assessment [HOMA][30]), the association between insulin resistance and the I allele has been reported in non–insulin-treated NIDDM patients, but not in a group of nondiabetic subjects.[31] The investigators speculated that the lack of association of ACE genotype with insulin sensitivity in nondiabetic subjects may imply an interaction between diabetes and the renin-angiotensin system in determining insulin sensitivity or may simply relate to the use of the HOMA model, which is not able to detect the difference in nondiabetic subjects. By using the glucose clamp technique, we demonstrate the association between the I allele and insulin resistance in glucose-tolerant and normotensive subjects.

Our observations are consistent with previous reports that exogenous infusion of angiotensin II increases insulin sensitivity in both glucose-tolerant and NIDDM patients.[32-35] Compared with subjects without the I allele (D/D), subjects with the I allele have lower plasma ACE levels, which produces the lower angiotensin II levels associated with insulin resistance, as observed in the present study. Since D/D subjects have a higher ACE level,[4] which in turn may produce a higher angiotensin II level, they may become more insulin-sensitive. Therefore, our data support the chronic effect (more insulin-sensitive) of angiotensin II in connection with the D/D genotype, compared with previous studies for the acute effect (also more insulin-sensitive) by infusion of angiotensin II. The study with acute angiotensin II infusion[33] suggested that the increase in glucose disposal is due to vascular alterations in organ blood flow: a decrease in renal blood flow that resulted in a reduced insulin clearance and a dose-related increase in femoral blood flow that resulted in an increase of tissue extraction of glucose. In

Table 2. Clinical Features by ACE Genotype

Feature	D/D			D/I + I/I		
	Mean	SE	%	Mean	SE	%
No. of subjects	8			16		
Age (yr)	32	3		31	2	
Gender (female, n)	5		63	11		69
Family history of diabetes (n)	4		50	7		44
Family history of hypertension (n)	5		63	7		44
Body mass index (kg/m^2)	28.2	2.1		29.1	1.3	
Waist to hip ratio	0.835	0.025		0.846	0.017	
Systolic blood pressure (mm Hg)	112	4		115	3	
Diastolic blood pressure (mm Hg)	71	3		71	2	

Table 3. OGTT Results by ACE Genotype

Parameter	D/D	D/I + I/I
No. of subjects	8	16
Plasma glucose (mmol/L)		
Fasting	4.60 ± 0.12	4.61 ± 0.09
30 min	6.54 ± 0.41	6.73 ± 0.35
60 min	6.54 ± 0.70	6.79 ± 0.37
90 min	5.37 ± 0.54	6.49 ± 0.21
120 min	5.95 ± 0.41	5.69 ± 0.14
Plasma insulin (pmol/L)		
Fasting	53 ± 18	78 ± 16
30 min	286 ± 88	550 ± 112
60 min	362 ± 75	546 ± 104
90 min*	250 ± 43	515 ± 69
120 min	370 ± 144	446 ± 63
Insulin area under the curve†	33,300 ± 8,114	56,200 ± 8,148
Stimulated plasma insulin (pmol/L)		
30 min	233 ± 71	472 ± 99
60 min	309 ± 59	468 ± 93
90 min‡	197 ± 33	436 ± 59
120 min	317 ± 127	368 ± 59

*P = .008, †P = .022, ‡P = .005: Mann-Whitney test.

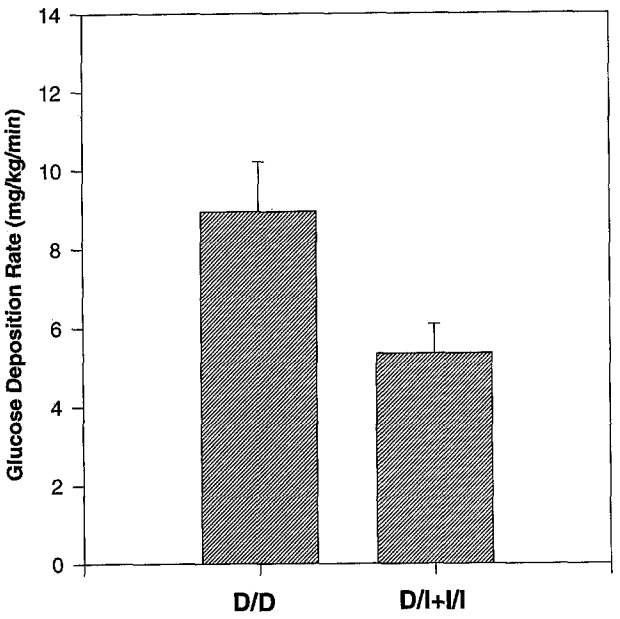


Fig 3. Glucose deposition rate during euglycemic clamp. Results are the mean ± SE; $P = .020$ by unpaired Student's t test.

addition to this hemodynamic mechanism, angiotensin II itself appears to have a direct biochemical effect on insulin sensitivity via various intracellular pathways that are involved in glucose utilization.[36-40] However, we do not know whether the chronic effect of angiotensin II, as a result of the D/D genotype, is mediated through the same mechanism as observed from the acute infusion of angiotensin II.

In the present study, we provide the first report of an association between the I allele at the ACE gene locus and insulin resistance by glucose clamp technique in glucose-tolerant and normotensive African-Americans. Since coronary artery disease and diabetic nephropathy are associated with the D allele,[6,7,14] the insulin resistance that is mediated through the I allele and higher angiotensin II level does not play a role in the pathogenesis of these two disease states. The role of the I allele in the pathogenesis of NIDDM remains to be determined.

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