DD Angiotensin-Converting Enzyme Gene Polymorphism Is Associated With Endothelial Dysfunction in Normal Humans

Robert Butler, Andrew D. Morris, Brian Burchell, Allan D. Struthers

Abstract—A polymorphism within the angiotensin-converting enzyme (ACE) gene may increase the risk of myocardial infarction in individuals previously thought to be at low cardiovascular risk. The mechanism through which it exerts this effect is unknown but may be due to increased angiotensin II–induced nitric oxide (NO) breakdown and/or reduced bradykinin-mediated NO release. We investigated whether endothelial function was different between different ACE genotypes. We performed a cross-sectional study comparing the endothelial function of the 3 genotypes (II: n=25; ID: n=31; DD: n=12). Mean±SD ages of the subjects were 24±4 (II), 25±6 (ID), and 25±6 (DD) years. We assessed the impact of the genotypes on endothelial function and found that the DD genotype was associated with a significant blunting in endothelial-dependent vasodilatation (forearm blood flow data are presented as mean±SD ratio of blood flow in response to 3 incrementally increasing doses of each vasoactive agent in the test arm to blood flow in the control arm; the comparison is between DD versus ID versus II; the P value is an expression of an overall difference by ANOVA, and the 95% CIs are of a pairwise comparison between genotypes): acetylcholine, 2.88±1.45 versus 3.81±1.93 versus 4.23±2.37 (P=0.002; 95% CI [II versus ID], −0.19 to 0.91; 95% CI [II versus DD], 0.36 to 1.80; 95% CI [ID versus DD], 0.02 to 1.42). There was also a significant difference with the endothelial-independent vasodilator sodium nitroprusside, with values of 2.11±1.00 versus 2.55±1.36 versus 2.75±1.18 (P<0.05; 95% CI [II versus ID], −0.15 to 0.51; 95% CI [II versus DD], 0.03 to 0.89; 95% CI [ID versus DD], −0.13 to 0.71), but not with verapamil. There was no effect of the ACE genotype on endothelial-dependent or -independent vasoconstrictors Nω-monomethyl-L-arginine or noradrenaline. Investigating the effects of cigarette smoking on each genotype demonstrated that for II and DD genotypes, acetylcholine responses were further blunted if subjects smoked. These data demonstrate that the DD ACE genotype in a young population is associated with a blunting of stimulated endothelial NO and donated NO responses but not to non-NO vasodilators or vasoconstrictors. (Hypertension. 1999;33:1164-1168.)

Key Words: angiotensin-converting enzyme ■ endothelium ■ nitric oxide ■ genes

Angiotensin-converting enzyme (ACE) is a metallopeptidase that governs the conversion of angiotensin I to angiotensin II and the degradation of bradykinin.1 In 1990, an insertion/deletion polymorphism within the ACE gene was discovered that accounts for almost half the variance of serum ACE concentrations in the normal population.2

The relevance of the ACE polymorphism was uncertain until the Etude Cas-Temoin de l’Insuffisance du Myocardie (ECTIM) Study3 showed that those who were homozygous for the deletion (D allele) were at increased risk of myocardial infarction compared with those homozygous for the insertion (I allele). Subsequently, the polymorphism has been reported to be a risk factor for myocardial infarction, coronary artery spasm, and left ventricular dysfunction. However, a number of published reports have found the opposite result for almost every clinical association, and we have recently reviewed the contentious role of the ACE genotype in cardiovascular disease.4

The hypothesis therefore arose that increased ACE activity might be a risk factor for myocardial infarction. This hypothesis was given added credence by the fact that enalapril reduced the incidence of myocardial infarction in both the Study of Left Ventricular Dysfunction (SOLVD) and the Survival and Ventricular Enlargement (SAVE) Study.5,6

Most studies of this topic involve case-control studies, but matching all variables is difficult. An alternative way to examine this is to investigate whether individuals with the DD genotype have endothelial dysfunction.

Endothelial dysfunction precedes macrovascular disease in human models7 of atherogenesis. The changes in ACE activity associated with the D allele could affect endothelial function by 2 potential mechanisms. First, increases in ACE conversion and therefore increase the generation of superoxide anions that degrade nitric oxide (NO). Second, increased

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From the University Department of Clinical Pharmacology and Therapeutics (R.B., A.D.S.); University Department of Medicine (A.D.M.); The Diabetes Center (A.D.M.); and University Department of Molecular Pathology (B.B.), Ninewells Hospital and Medical School, Dundee, UK.

Correspondence to Dr. R. Butler, University Department of Clinical Pharmacology and Therapeutics, Ninewells Hospital and Medical School, Dundee, UK DD1 9SY. E-mail r.butler@btinternet.com

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ACE levels may increase bradykinin degradation and therefore reduce bradykinin-induced NO effects.

Another major factor in atherogenesis is cigarette smoking. We have therefore also investigated how cigarette smoking influences the relationship between the ACE genotype and endothelial dysfunction.

Methods

Sixty-eight normal volunteers gave written informed consent to participate in this study, which was approved by the Tayside Medical Ethics Committee. None had evidence of cardiovascular disease as determined by history or clinical examination.

The study population was drawn from 2 sources: (1) those native to Dundee and therefore with the genetic background of northeast Scotland and (2) a more mobile and heterogeneous population of students attending the local university. Sixty-five of the 68 volunteers described their origins as white and of United Kingdom or Irish descent. Two were from the Indian subcontinent, and the final subject was Malaysian.

Study Protocol

After initial screening, each subject attended two 3-hour study mornings to evaluate vascular function. On each study morning, after a 12-hour overnight fast (water was permitted), endothelial function was assessed by bilateral forearm venous occlusion plethysmography with intra-arterial infusion of endothelial-dependent (acetylcholine) and endothelial-independent (sodium nitroprusside and verapamil) vasodilators.

A second study day evaluated the vascular responses to aminoglycoside vasodilators. On the second study day, we investigated the effect of endothelial-dependent intra-arterial vasoconstriction using L-NMMA (60, 120, and 240 μmol/mL) and the effect of endothelial-independent vasoconstriction using norepinephrine (1, 2, and 4 μmol/mL). The same drug order was used each time for study days 1 and 2.

BLOOD sampled at the screening visit for genotyping and for serum urea, creatinine, cholesterol, and plasma ACE. Urea, creatinine, and cholesterol were analyzed in-house on the day of the screening visit. Plasma ACE was analyzed as a single batch. Plasma ACE was measured with the use of a COBAS MIRA sigma test kit (Department of Biochemical Medicine, Ninewells Hospital and Medical School, Dundee, UK).

ACE Genotyping

The blood was collected in EDTA and stored at −20°C. DNA was extracted in a 300-μL reaction from a commercially available kit (Puregene, Gentra). The quantity of DNA was confirmed by spectrophotometer.

Polymerase Chain Reaction Conditions

A reaction volume of 50 μL consisted of 10 mmol dNTP; 5 pmol each of ACE 1, ACE 2, and ACE 3; 2.5 μL W-1 (1%); 1.5 mmol MgCl; 50 mmol KCl; 20 mmol Tris-HCl (pH 8.4); 1 U Taq DNA polymerase; and 0.5 μg DNA. The reaction was heat-started at 95°C for 1 minute, followed by 30 cycles of amplification (1 minute of denaturation [94°C], 1 minute of annealing [50°C], and 30 seconds of extension [72°C]).

The polymerase chain reaction was performed with the use of oligonucleotides as described by Shanmugan et al in a modification of the original method of Rigat et al, which avoids mistyping 5% of ID genotypes as DD genotypes. The primers result in 2 amplified products: 84 bp (D allele) and 65 bp (I allele). These were run on a 12% polyacrylamide gel, then stained with ethidium bromide and visualized with ultraviolet light.

Statistical Analysis

Venous occlusion plethysmography produces 2 discrete values of blood flow in milliliters per 100 mL forearm volume per minute, one

<table>
<thead>
<tr>
<th>TABLE 1. Baseline Data, Including Blood Pressure, Cholesterol, Plasma ACE, and Basal Blood Flow</th>
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</thead>
<tbody>
<tr>
<td><strong>Baseline Variables</strong></td>
</tr>
<tr>
<td>Smokers</td>
</tr>
<tr>
<td>Mean No. of cigarettes per day</td>
</tr>
<tr>
<td>Mean duration of smoking, y</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
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<tr>
<td>HDL cholesterol, mmol/L</td>
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<tr>
<td>Serum ACE, IU</td>
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<tr>
<td>Baseline blood flow, mL/(100 mL - min)</td>
</tr>
<tr>
<td>Day 1</td>
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<tr>
<td>Day 2</td>
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</tbody>
</table>

Values are mean± SD.
for each arm. Blood flow changes over time, but the assumption is made that flow in each arm at rest will be equivalent. Therefore, all plethysmographic data are presented as a ratio of blood flow in the test arm to that in the control arm. Blood flow measurements were compared between genotypes by a general linear model by multivariate ANOVA (MANOVA), with genotype and dose as factors. The Bonferroni method was then used for calculating 95% CIs after analysis by ANOVA (II versus ID, II versus DD, and ID versus DD), thus enabling the reader to locate the origin for any significant difference.

The figures present the data as mean±SE for visual clarity. Differences were considered statistically significant at P<0.05.

**Results**

Sixty-eight male subjects with mean±SD age of 24±4 (II), 25±6 (ID), and 25±4 (DD) years and a body mass index of 23.0±2.5 (II), 23.3±2.7 (ID), and 23.3±1.6 (DD) kg/m² completed the study. There were no differences in blood pressure, cholesterol, or HDL cholesterol between the 3 study groups (Table 1). There was a significant increase in serum ACE level with genotype (Table 1).

**Frequency of D/I Alleles and Genotypes**

The percentages of D/I alleles were 40% and 60%, respectively, resulting in a genotypic distribution as follows: DD, 18%; ID, 45%; and II, 36%.

**Forearm Blood Flow**

**Baseline Blood Flow**

There were no significant differences in baseline blood flow between different genotypes on either study day (Table 1).

**Vasodilators**

We found that the DD genotype was associated with a significant blunting in endothelial-dependent vasodilatation: acetylcholine (mean±SD, DD versus ID versus II), 2.88±1.45 versus 3.81±1.93 versus 4.23±2.37 (P=0.002; 95% CI [II versus ID], −0.19 to 0.91; 95% CI [II versus DD], 0.36 to 1.80; 95% CI [ID versus DD], 0.02 to 1.42). There was also a significant difference with the endothelial-independent vasodilator sodium nitroprusside, with values of 2.11±1.00 versus 2.55±1.36 versus 2.75±1.18 (P<0.05; 95% CI [II versus ID], −0.15 to 0.51; 95% CI [II versus DD], 0.03 to 0.89; 95% CI [ID versus DD], −0.13 to 0.71), but not with verapamil, with values of 4.48±2.24 versus 4.96±3.73 versus 5.08±4.48 (P=0.07; 95% CI [II versus ID], −1.31 to 0.57; 95% CI [II versus DD], −1.67 to 0.79; 95% CI [ID versus DD], −1.25 to 1.13) (Figure 1).

The final data point in Figure 1 in the panel representing the verapamil dose-response curve shows an anomalous point, which is due to a single large value. After we reviewed the original data, this subject’s data cannot be justifiably excluded. However, the outcome (no significant difference) is unchanged whether this subject is included or excluded.

**Vasoconstrictors**

There was no effect of the ACE genotype on endothelial-dependent or -independent vasoconstrictors: L-NMMA, 0.80±0.22 versus 0.82±0.23 versus 0.83±0.18 (P=0.29; 95% CI [II versus ID], −0.08 to 0.04; 95% CI [II versus DD], −0.11 to 0.06; 95% CI [ID versus DD], −0.09 to 0.06); norepinephrine, 0.64±0.23 versus 0.64±0.18 versus

![Figure 1](http://hyper.ahajournals.org/issue/2/5/1166/figures/001-f1.png)

**Figure 1.** Dose-response curve for acetylcholine, nitroprusside, and verapamil for the II, ID, and DD genotypes (mean±pooled SE).

0.66±0.12 (P=0.46; 95% CI [II versus ID], −0.04 to 0.08; 95% CI [II versus DD], −0.08 to 0.09; 95% CI [ID versus DD], −0.09 to 0.07) (Figure 2).

**Smoking and Endothelial Function in Each Genotype**

Smoking was significantly associated with some blunting of NO-mediated vascular responses in all 3 genotypes. Acetylcholine responses were significantly blunted in both II and DD smokers. L-NMMA responses were significantly blunted in ID smokers (Table 2).

**Discussion**

This study demonstrates that an insertion/deletion polymorphism in the ACE gene is associated with significant blunting of NO vasodilatory responses in young healthy men. This study also describes a novel observation in association
with the D allele that endothelial-independent vasodilatation (nitroprusside) is also blunted. A similar effect in young cigarette smokers has been described previously,14 in which blunted responses to both flow-mediated vasodilatation and sublingual glyceryl trinitrate were observed. We have previously found lisinopril to improve both acetylcholine and nitroprusside responses in hypercholesterolemic subjects.15 Our observation that the D allele has no effect on the control non-NO vasodilator verapamil suggests that this is a specific effect limited to NO pathways.

Previous work on endothelial function has revealed the following. In vitro data16 show that human internal mammary arteries demonstrate blunted endothelial-dependent vasodilatation and augmented endothelial-dependent vasoconstriction in subject with established coronary disease. In vivo, Celermajer et al17 found no differences between genotypes with brachial artery ultrasound using flow-mediated dilatation and glyceryl trinitrate. More recently, Perticone et al18 demonstrated that the DD genotype is associated with significant blunting of vascular responses to intra-arterial acetylcholine in patients with never-treated hypertension, with no difference in the nitroprusside response.

Our finding of a blunted vasodilator response to acetylcholine and nitroprusside disagrees with that of Celermajer et al,17 although Celermajer used flow-mediated dilatation, in contrast to our methods. Methodological differences may explain these disparate findings. First, flow-mediated dilatation is associated with changes in the magnitude of 10% rather than the 400% we observed with venous occlusion plethysmography, and it is easier to note subtle changes with a large rather than a small signal. Second, flow-mediated dilatation measures conduit artery blood flow rather than flow through the skeletal muscle microcirculation, as plethysmography does.

The L-NMMA responses did not appear different between different genotypes, while the acetylcholine and nitroprusside responses were different. It is possible that tonic NO production is essentially normal, but stimulated NO responses are not. An alternative explanation is methodological, ie, vasodilators produce increases of 200% to 400% in blood flow, whereas L-NMMA only produces a 20% fall, and it is much easier to note a subtle difference between different genotypes with a large signal.

There are 2 possible explanations for this link between the ACE genotype and arterial function: (1) ACE governs the degradation of bradykinin, and therefore increased ACE activity could decrease bradykinin bioactivity, which in turn could reduce receptor-mediated release of NO. Circumstantial evidence for this arises from data in which ACE inhibi-

TABLE 2. Effects of Cigarette Smoking on Vascular Responses Within Each Genotype

<table>
<thead>
<tr>
<th>Genotype/NO Vasoactive Agent</th>
<th>Nonsmoker</th>
<th>Smoker</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>Acetylcholine</td>
<td>4.9±2.5</td>
<td>3.4±1.9</td>
</tr>
<tr>
<td></td>
<td>Nitroprusside</td>
<td>3.0±1.2</td>
<td>2.4±1.1</td>
</tr>
<tr>
<td></td>
<td>Verapamil</td>
<td>4.7±2.3</td>
<td>4.2±2.2</td>
</tr>
<tr>
<td></td>
<td>L-NMMA</td>
<td>0.76±0.22</td>
<td>0.85±0.21</td>
</tr>
<tr>
<td></td>
<td>Norepinephrine</td>
<td>0.59±0.24</td>
<td>0.73±0.19</td>
</tr>
<tr>
<td>ID</td>
<td>Acetylcholine</td>
<td>3.7±2.0</td>
<td>3.9±1.9</td>
</tr>
<tr>
<td></td>
<td>Nitroprusside</td>
<td>2.5±1.1</td>
<td>2.6±1.6</td>
</tr>
<tr>
<td></td>
<td>Verapamil</td>
<td>5.1±4.7</td>
<td>4.8±2.3</td>
</tr>
<tr>
<td></td>
<td>L-NMMA</td>
<td>0.77±0.23</td>
<td>0.88±0.21</td>
</tr>
<tr>
<td></td>
<td>Norepinephrine</td>
<td>0.61±0.20</td>
<td>0.68±0.16</td>
</tr>
<tr>
<td>DD</td>
<td>Acetylcholine</td>
<td>3.3±1.4</td>
<td>2.1±1.1</td>
</tr>
<tr>
<td></td>
<td>Nitroprusside</td>
<td>2.1±1.1</td>
<td>2.0±0.9</td>
</tr>
<tr>
<td></td>
<td>Verapamil</td>
<td>4.7±1.9</td>
<td>5.8±7.4</td>
</tr>
<tr>
<td></td>
<td>L-NMMA</td>
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<tr>
<td></td>
<td>Norepinephrine</td>
<td>0.66±0.12</td>
<td>0.63±0.08</td>
</tr>
</tbody>
</table>

Values are mean±SD.
tion increases bradykinin-mediated NO effects.\textsuperscript{19} (2) The second possible mechanism is that angiotensin II increases levels of superoxide through increased activity of NADH/NADPH oxidase activity,\textsuperscript{20} which can reduce the bioactivity of NO.\textsuperscript{21} However, the literature is variable on whether angiotensin II effects are increased in the \textit{DD} ACE genotype or not; there are data to both support and refute this hypothesis.\textsuperscript{22–24}

\textbf{Study Limitations}

Our sample was drawn from students attending the local university, and the distribution of the I/D alleles differs slightly from observations in other large European populations, suggesting that our study may not represent a true population sample. However, we are certain that the plasma ACE level increases with the D allele, suggesting that despite its apparent heterogeneity, the phenotypic expression is maintained.

The blunting of endothelial function by smoking is particularly evident in the \textit{II} and \textit{DD} groups. However, it may be difficult to draw firm conclusions because of the relatively small number of smokers in the \textit{DD} group. The magnitude of the effect of smoking on acetylcholine responses is almost identical in the 2 extremes of the genotype (\textit{II} and \textit{DD}). This suggests that the ACE genotype and smoking produce additive rather than synergistic effects on endothelial dysfunction.

\textbf{Conclusion}

These data suggest that the \textit{DD} ACE genotype is associated with arterial dysfunction limited to NO pathways. Interestingly, this effect is already evident in young men. Although the mechanism is unclear, it may be amenable to therapy with ACE inhibitors or angiotensin receptor antagonists.

\textbf{Acknowledgments}

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\textbf{References}


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