Angiotensin I-Converting Enzyme Genotype Influences Arterial Response to Injury in Normotensive Rats

Mireille Challah, Eric Villard, Monique Philippe, Antoine Ribadeau-Dumas, Bruno Giraudieu, Philip Janiak, Jean-Paul Vilaine, Florent Soubrier, Jean-Baptiste Michel

Abstract—Two normotensive strains of rat, the Lou and Brown Norway (BN) strains, have contrasting levels of plasma angiotensin–converting enzyme (ACE). To investigate the degree of genetic determination of ACE expression, a polymorphic marker of the ACE gene was analyzed in inbred rats of the two strains. The two inbred strains were shown to bear different alleles for a polymorphic marker at the ACE gene. The segregation of the alleles of this marker and the plasma ACE levels were studied in a group of F2 rats issued from a cross between Lou and BN rats. The degree of genetic determination of plasma ACE activity was estimated to be 94% in the F2 cohort. The ACE locus accounts for 74% of total plasma ACE variance. ACE activity and mRNA expression in lungs were also genetically determined. The difference observed in ACE mRNA accumulation in the lungs between the two strains was due to a difference in the transcriptional rate of the ACE gene, as shown in nuclear run–on experiments. No differences were observed in arterial blood pressure of homozygous F2 progeny. In these animals, ACE genotype did not interfere with the pressor or the depressor responses to ACE-dependent vasoactive peptides. There was a significant effect of strain on constitutive or inducible membrane or soluble ACE activity in primary cultures of vascular cells. Neointima formation in the carotid artery 14 days after balloon injury was also influenced by the genotype in F2 homozygous progeny, whereas the medial area was not. These results demonstrate that there is a close relationship between the genetically determined ACE expression and the inducibility of the ACE gene. The degree of genetic determination of ACE expression in inbred rat strains offers a unique opportunity to study the interaction between genetic and environmental determinants of ACE expression and its involvement in response to experimental cardiovascular and renal injury. (Arterioscler Thromb Vasc Biol. 1998;18:235-243.)

Key Words: angioplasty • angiotensin • cells • gene • genetic

The ACE is constitutively expressed in endothelial cells lining blood vessels and can be induced in SMCs and fibroblasts in response to cardiovascular injuries and hormones such as fibroblast growth factor or dexamethasone.1,2 Several lines of data obtained in the rat suggest that nonendothelial ACE plays an important role in pathological processes of the vascular wall.3–5 Indeed, ACE inhibition decreases neointimal proliferation after balloon injury of the carotid wall,6 and ACE cDNA transfection of SMCs in an injured carotid artery increases cell proliferation.7 A polymorphism of the ACE gene in humans is responsible for large interindividual variations of plasma ACE levels.8 Combined segregation and linkage analyses showed that the ACE locus is responsible for the major part of this genetically determined variation.7 The I/D polymorphism explains the highest part of plasma ACE variance in French normal nuclear families, but genetic analysis supports a complex model with two putative functional variants linked to the ACE gene.10 Nevertheless, the molecular mechanism explaining the contrasting plasma ACE levels remains unknown. Furthermore, cell and tissue ACE levels have also been shown to be modulated by ACE gene polymorphism in humans.11,12 Since Cambien et al13 first described a significant association between the I/D polymorphism and myocardial infarction, many studies have tried to examine the association of the ACE genotype, not only with ischemic heart disease14–17 but with a variety of conditions ranging from hypertension18,19 to coronary artery restenosis,14,20,21 cardiac hypertrophy,16,22,23 pulmonary hypertension,24 diabetes,25 and nephropathy.26–28 The results of these studies are contradictory and stress the importance of experimental models for testing the impact of the ACE gene polymorphism and its associated modulation of ACE expression on the cardiovascular and renal responses to these pathological processes.

The ACE locus is well characterized in rats since the region of chromosome 10, which contains the ACE gene, is involved in BP regulation in rats,29,30 but no genetic linkage was found between the ACE gene and BP.31 A microsatellite marker was identified in the ACE gene, within intron 13, allowing distinction of ACE alleles between rat strains.29,30

We investigated the plasma ACE levels in various strains of rats and studied the degree of genetic determination of ACE expression in a cross between two strains of normotensive rats...
with contrasting levels of plasma ACE. In this study we determined at which step ACE gene expression was modulated by the polymorphism between the two strains of rats. We demonstrated a significant effect of strain on ACE activity in primary cultures of ECs, SMCs, and fibroblasts. Finally, we tested the hypothesis that the ACE alleles cosegregated with contrasting responses to carotid injury in a cross between these strains with contrasting plasma and tissular ACE.

Methods

Genetic Characterization of ACE Expression

Measurement of Plasma ACE Activity
Plasma ACE activity was measured in different normotensive inbred strains of rats (3 months old), Lou, BN, Lewis, Fisher 344, and Long Evans (n = 10 for each strain), using a previously described fluorometric assay.32

Crossbreeding Procedures and Genotyping
Five female Lou rats (CNRS, Orleans, France) and five male BN rats (Iffa Credo, L’Abresle, France) were mated to produce F1 hybrids. Ten male and 10 female rats of the F1 generation were mated to produce the F2 cohort.

The polymerase chain reaction primers and the procedure used to amplify the microsatellite located at the 5' end of intron 13 inside the rat ACE gene and characterized by a (CA)₆ repeat were described by Hilbert et al.29

Measurement of Membrane ACE Activity
Rats from the F2 cohort, eight homozygous for the Lou microsatellite allele (LL F2) and eight homozygous for the BN microsatellite allele (BB F2), were anesthetized with sodium pentobarbital (60 mg/kg). Lungs were quickly excised, rinsed, and frozen in liquid nitrogen.

The procedure followed for the care and killing of studied animals was in accordance with the European Community Standards on the Care and Use of Laboratory Animals (Ministère de l’Agriculture, France; authorization 00577).

Lungs were homogenized in 10 vol of cold Tris-HCl buffer. The supernatant was incubated with Z-PHL, a synthetic ACE substrate.32 Protein concentration was determined by the Bradford method.

Analysis of Lung ACE mRNA by Northern Blot
Total RNA from BB F2 and LL F2 lungs was extracted by the Chomczynski and Sacchi method.31 Forty micrograms of total lung RNA was used for Northern blotting.34 RNA blots were probed with ATR31 rat ACE cDNA20 labeled with α-32P-dCTP. Prehybridization, hybridization, and washing were performed in standard conditions.

The lung ACE mRNA was normalized to GAPDH mRNA by rehybridization of the blot with a human GAPDH cDNA probe that cross-hybridizes to rat GAPDH mRNA. The radiolabeled and hybridized probe was quantified by the β Image 1200.26

Run-On Assay
All procedures for isolation of nuclei from lungs were performed on ice at 4°C. Lung nuclei from BB F2 and LL F2 rats were isolated as described by Boggaramadn Mendelson.37 Each transcription elongation reaction was carried out in the presence of 4 × 10⁻⁵ isolated nuclei as described by Mak et al38 with slight modifications.

Equivalent amounts of radioactive RNA (7.5 × 10⁶ cpm) from BN and Lou lungs were added to slot blots, three slots on the same blot, on which 10 µg of denatured plBluescript (as control), 10 µg of plasmid containing insert of rat ACE cDNA, or 10 µg of rat GAPDH cDNA had been immobilized to a nylon filter. After hybridization, the blots were washed,37 then exposed to Kodak Biomax MS film with an intensifying screen at ~80°C for 1 to 3 days.

Vascular Cell Phenotypes
The three cell types from aortae (ie, ECs from intima, SMCs from media, and fibroblasts from adventitia) of both Lou and BN inbred strains were isolated and cultured as described by Battle et al.33 The purity of the cultures was assessed using immunohistological and morphological criteria. ECs were stained for Von Willebrand factor and by RECA-1 (a monoclonal antibody specific for rat endothelial cells, kindly provided by Dr A.M. Duijvestijn, Maastricht, The Netherlands). At confluence ECs exhibited a typical cobblestone pattern. SMCs were characterized using α-actin antibodies.31 At confluence SMCs were typically fusiform. Fibroblasts were negative to the previously cited stainings and at confluence did not exhibit a specific organized pattern. Morphological criteria are described in detail elsewhere.32

Animals were pretreated during 1 week with a high-salt diet (1% NaCl in drinking water), then binephrectomized 24 hours before the experiment to suppress the endogenous renin-angiotensin system. Rats were anesthetized with pentobarbital (60 mg/kg) for catheter implantation.

Plasma Renin-Angiotensin System
Rats were killed by decapitation. Measurement of plasma renin activity prevented the use of anesthesia. Plasma renin activity was determined by radioimmunoassay of angiotensin I generated in vitro.43 Angiotensinogen concentration was determined in similar conditions by adding an excess of mouse renin.44 Plasma angiotensin I and II concentrations were measured according to the procedure of Nussberger et al43 with slight modifications.

Response to Exogenous Peptides
In Vivo Experiments
Blood pressure was measured by the tail cuff method in Lou and BN rats and in homozygous F2 progeny (LL F2 and BB F2 rats). Body weight and heart weight were assessed in the same animals.

Plasma Renin-Angiotensin System
Rats were killed by decapitation. Measurement of plasma renin activity prevented the use of anesthesia. Plasma renin activity was determined by radioimmunoassay of angiotensin I generated in vitro.43 Angiotensinogen concentration was determined in similar conditions by adding an excess of mouse renin.44 Plasma angiotensin I and II concentrations were measured according to the procedure of Nussberger et al43 with slight modifications.

Response to Exogenous Peptides
Intravenous injections of angiotensin I and bradykinin were performed in two groups of conscious, catheterized homozygous (n = 16) F2 rats. Rats were anesthetized with pentobarbital (60 mg/kg) for catheter implantation.

Animals were pretreated during 1 week with a high-salt diet (1% NaCl in drinking water), then binephrectomized 24 hours before the experiment to suppress the endogenous renin-angiotensin system. Rats were anesthetized with pentobarbital (60 mg/kg) before bi-nephrectomy. The experimental protocol is explained elsewhere in detail.39 Angiotensin I was successively injected at 50, 100, and 300 mg/kg IV every 15 minutes. Then, bradykinin was successively

**Selected Abbreviations and Acronyms**

ACE = angiotensin I-converting enzyme
ANOVA = analysis of variance
BN = Brown Norway
BP = blood pressure
EC = endothelial cell
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
I/D = insertion/deletion
SMC = smooth muscle cell
Z-PHL = Z-phenylalanyl-histidyl-leucine

**References**
Response to Balloon-Induced Arterial Injury

To determine whether the genotype of ACE would influence neointima formation, homozygous F2 rats for the ACE microsatellite (n=13) were anesthetized (5 mg/kg IP acepromazine, 100 mg/kg IP ketamine), and a 2F balloon embolectomy catheter was introduced through the external branch of the left common carotid artery into the aortic arch. The vascular injury of the left carotid artery was performed by slowly pulling out the inflated balloon as described by Clowes et al.47 The procedure was repeated three times. The injury was applied without knowing the animal’s genotype.

Fourteen days after intimal injury, rats were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg). The carotid artery removal procedure is described in detail elsewhere.48 Only the portion of the carotid artery that did not show any evidence of endothelial regeneration (positive staining after Evans blue injection) was used for histomorphometric studies. The portion was divided into four segments (2.5 mm long) and embedded in paraffin. Nonserial cross sections (5 μm) were prepared and stained with orcein to measure medial and neointimal cross-sectional areas. The distance between two cross sections was 50 μm. All histomorphometric analyses were performed in a double-blind manner with a computerized image-analysis system with 256 levels of gray and a 512×512 pixel grid (HISTO software). This system allows an overall analysis of the cross section. The internal elastic lamina was used as the border to distinguish the neointima from the media. For each carotid artery, neointimal and medial cross-sectional areas were averaged from the analysis of 12 cross sections (three cross sections per segment). The ratio of the cross-sectional area of the neointima to the cross-sectional area of the media was then calculated.

Cross sections were stained with red nucleus dye to quantify intimal and medial nuclear densities. Nuclear counting was performed manually, using a grid in the microscope eyepiece at a magnification of ×400, by an observer blinded to the genotype of the samples. Nuclear counts were made on four fields per section, chosen at random at four cardinal points. Four sections were used per sample (n=8).

Statistical Analysis

Data are expressed as mean±SEM. Statistical significance of differences between strains was estimated by one-way ANOVA, followed by Bonferroni’s test. A three-way (fields, sections, and rats) nested ANOVA was performed to provide evidence that differences observed in the density of intimal cell nuclei during intimal proliferation were related to the experimental genetic model rather than to the variance of other steps of analysis. Covariance analysis was used to test the effect of dexamethasone and the effect of ACE genotype on cellular ACE activity and the interaction between dexamethasone induction and the degree of genetic determination of ACE expression in primary vascular cell cultures. Statistical significance was reached when P was <.05.

The degree of genetic determination of plasma ACE concentration was estimated as (Vt–Vc)/Vc, where Vc was the total variance of the phenotype in the F2 cohort and Vt was a pooled estimate of the environmental phenotype variance in the Lou, BN, and F1 cohorts. The proportion of plasma ACE variance in F2 rats that could be attributed to the ACE microsatellite locus in the F2 cohort was deduced from the calculation by ANOVA of the sum of squares due to the genotypes compared to the sum of squares of plasma ACE in the F2 rats. The possibility of deviation from additivity of allelic effects (homozygous mean at the midpoint of homozygous means) was tested using a regression analysis of plasma ACE levels on the numbers of BN alleles.

Results

Genetic Determination of ACE Expression in Normotensive Rats

Plasma ACE Activity in Various Inbred Rat Strains

As shown in Fig 1, plasma ACE activity was homogeneous within each inbred strain, but differences were observed between strains. There were no differences according to age or sex in mature animals from 1 to 9 months old rats (data not shown). BN and Lou rats showed a twofold difference in ACE plasma activity and were chosen for further analysis.

ACE Genotype Effect on Plasma ACE Determination

The ACE microsatellite was polymorphic between the BN and Lou strains, and the segregation of the BN alleles (B) and of the Lou alleles (L) was analyzed in F1 and F2 progeny. All the F1 progeny were heterozygous for the BN and Lou alleles (BL). The distribution of the ACE genotypes (BB, BL, and LL) in the F2 population was in accordance with Mendelian segregation.

Mean plasma ACE activity was measured in the F0, F1, and F2 groups (Table 1). The F1 generation exhibits plasma ACE activity intermediate between the two parental strains, Lou and BN, with a very small variance. In total, 40 F1 progeny were produced (19 males and 21 females). There were no sex differences (108.7±1.6 for the males versus 106.2±1.6 nmoles PHL/mL · min−1 for the females). In the F2 progeny, plasma ACE activity was compared among the three groups defined by ACE microsatellite genotype (LL, BL, and BB). Plasma ACE activity was not significantly different between the LL F2 and the LL F0 progeny, between the BL F2 and the BL F1 progeny, and between the BB F2 and the BB F0 progeny. The degree of genetic determination (ratio of genetic to total

<table>
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<tr>
<th>Strains</th>
<th>Plasma ACE Activity (nmoles PHL/mL · min−1)</th>
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<tbody>
<tr>
<td>Lou</td>
<td>68.3±1.3 (n=15)</td>
</tr>
<tr>
<td>Lewis</td>
<td>136.1±4.1 (n=15)</td>
</tr>
<tr>
<td>LE</td>
<td>107.5±1.1 (n=40)</td>
</tr>
<tr>
<td>Fisher</td>
<td>58.7±2.7 (n=22)</td>
</tr>
<tr>
<td>BN</td>
<td>106.1±2.2 (n=47)</td>
</tr>
<tr>
<td>BN</td>
<td>144.5±2.6 (n=31)</td>
</tr>
</tbody>
</table>

TABLE 1. Plasma ACE Activity in F0, F1, and F2 Progeny of Lou and BN Crossbreeding and Genotypes for the ACE Microsatellite Marker

Results are mean±SEM, and plasma ACE activity is in nanomoles per milliliter per minute.
variance) in the F2 cohort was estimated as 94% for plasma ACE activity. The ACE locus accounts for 74% of total plasma ACE variance (80% of genetic variance). No deviation from additivity of ACE polymorphism alleles on plasma ACE was observed.

Membrane ACE Activity and Expression in Lung Tissues of BN and Lou Rats

Lung membrane ACE activity was higher in BN (n = 8) than in Lou (n = 8) rats (890±64 versus 498±57 nmoles PHL/mg protein · min⁻¹, P < .01). The lung ACE mRNA level was also higher in BB F2 (n = 7) than in LL F2 (n = 7) progeny (P < .001) (Fig 2).

The transcription rate of the ACE gene in isolated nuclei from lung tissues, calculated from the ratio of transcription rate of the ACE gene to that of the GAPDH gene, was 1.8-fold higher in BB F2 (n = 8) than in LL F2 (n = 8) progeny (P < .01) (Fig 3).

ACE Phenotype in Cultured Arterial Cells

Results of analysis of covariance are summarized in Table 2 and Fig 4. All the slopes of the covariances between dexamethasone doses and ACE activity were significantly different from zero. There was no significant interaction between genotype and dexamethasone dose effects when membrane ACE activity of ECs and SMCs were assessed. In ECs we observed a genotype effect on the y intercept of covariance. When soluble ACE activity in the culture medium was assessed, a significant interaction between genotype and dexamethasone effects was observed in both ECs and SMCs. In fibroblasts, we observed a significant genotype-dexamethasone interaction when membrane ACE activity was measured, whereas no interaction was observed when it was assessed in the culture medium.

Vascular Response In Vivo

Pressure and Weights

Blood pressure was measured in 6-week-old Lou and BN rats and in 10-week-old homozygous F2 progeny. The Lou and BN rats were both normotensive, but the BP was significantly higher in the young BN rats (n = 8; 115.7±2.0 versus 128.7±2.5 mm Hg for Lou and BN, respectively; P < .001). No differences were observed in the BP between the BB F2 and the BB F2 rats (n = 10; 134.0±2.7 versus 134.4±2.9 mm Hg; not significant).

In the BN rats (n = 6), body and heart weights were significantly higher as compared to the Lou rats (n = 6), whereas no differences were detected between the ACE homozygous F2 progeny (n = 10) (Table 3).

Functional Responses

There were no differences in plasma renin activity between the BN (n = 8) and the Lou rats (n = 8). Similarly, no differences in

<table>
<thead>
<tr>
<th>Vascular Cells</th>
<th>ACE Activity</th>
<th>Difference Between Slopes y Intercept</th>
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<tbody>
<tr>
<td>ECs Membrane</td>
<td>NS</td>
<td>F = 68.7, P &lt; .001</td>
</tr>
<tr>
<td>Culture medium</td>
<td>F = 17.2, P &lt; .001</td>
<td></td>
</tr>
<tr>
<td>SMCs Membrane</td>
<td>NS</td>
<td>F = 183.1, P &lt; .001</td>
</tr>
<tr>
<td>Culture medium</td>
<td>F = 14.0, P &lt; .001</td>
<td></td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Membrane</td>
<td>F = 6.4, P &lt; .001</td>
</tr>
<tr>
<td>Culture medium</td>
<td>NS</td>
<td>F = 123.3, P &lt; .001</td>
</tr>
</tbody>
</table>

NS indicates not significant.

The equality of slopes test is used to assess interaction between genotype and dexamethasone dose effects. Only when there is no significant interaction can the y intercept be considered.
plasma angiotensinogen and angiotensin concentrations were detected (Table 4).

The basal level of blood pressure was not different between the two groups (LL F2, 131.0 ± 18.5; BB F2, 130.0 ± 23.4 mm Hg). Angiotensin I given at 50, 100, and 300 ng/kg elicited a dose-dependent increase in mean BP in the two homozygous F2 groups (Fig 5, A) (P < .0001). There was no significant difference in the response between the LL F2 (n = 8) and the BB F2 (n = 8) rats (F = 0.23, not significant).

Bradykinin administration at 1, 2.5, and 5 μg/kg induced a dose-dependent decrease in BP in the two homozygous F2 groups (Fig 5, B) (P < .0001). No differences in the response were observed between the LL F2 (n = 8) and the BB F2 (n = 8) rats (F = 0.71, not significant).

**Table 3. Body Weight and Heart Weight in F0 and ACE Homozygous F2 Progeny of Lou and BN Crossbreeding**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Homozygous LL</th>
<th>Homozygous BB</th>
<th>P†</th>
</tr>
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<tbody>
<tr>
<td>F0 (18 wk)</td>
<td>Body weight, g</td>
<td>228.5 ± 16.5</td>
<td>314.5 ± 7.2</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>670.0 ± 48.0</td>
<td>848.0 ± 26</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>F2 (10 wk)</td>
<td>Body weight, g</td>
<td>195.8 ± 7.3</td>
<td>197.9 ± 4.2</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>629.2 ± 17.2</td>
<td>653.1 ± 18.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS indicates not significant.

*Results are expressed as mean ± SEM.
†Based on ANOVA.

**Figure 4. Representation of covariance between dexamethasone doses and ACE activity in BN (•, —) and Lou (○, —) ECs and SMCs.** Three days after confluency was reached, cells were deprived of serum for 24 hours, then treated for 48 hours with the indicated dexamethasone concentrations. Top, ACE activity was measured on membrane cells. Bottom, Soluble ACE activity was measured in the culture medium. The genotype significantly influences the constitutive membrane expression in ECs, and there is a significant interaction between genotypes and dexamethasone doses on ACE activity in the culture medium of both ECs and SMCs.

**Figure 5. Pressor response to exogenous vasoactive peptides in rats submitted to bilateral nephrectomy.** A, Pressor response to an intravenous bolus injection of angiotensin I in LL F2 (□) and BB F2 (■) rats. B, Effects of an intravenous bolus injection of bradykinin on variation of mean BP in LL F2 (□) and BB F2 (■) rats. Values are means ± SEM. There were no significant differences in the pressor response due to genotype.

**Structure Response to Vascular Injury by Ballooning**

Fourteen days after injury of the carotid artery, we observed formation of a massive neointima in both homozygous F2 progeny. However, the extent of the arterial lesion was significantly influenced by the ACE genotype. Results are summarized in Fig 6. The neointima area and the neointima/media ratio were greater in the BB F2 group (n = 13) than in the LL F2 group (n = 11) (P < .0001), whereas media area remained unchanged (F = 2.4, NS). No differences were observed in the luminal (F = 0.3; NS) and external medial (F = 2.4, not significant) perimeters between the two groups. The density of intimal cell nuclei was significantly higher in BB F2 group than in the LL F2 group (P < .0001), whereas in the media the density of cell nuclei was not different between the two groups (F = 0.7, not significant). Nested ANOVA showed that only the ACE genotype significantly influenced the density of intimal cell nuclei (field effect, F = 0.8, not significant; section effect, F = 0.3, not significant).

**Discussion**

The steady level of plasma ACE, apparently weakly influenced by environmental factors in physiological conditions in rats and humans, allows the study of its degree of genetic determination by polymorphic markers on the genes controlling its level. In normotensive rats as well as in humans, we found a polymorphism of the plasma ACE concentration, likely resulting from a polymorphism of expression of the gene. Our study of different homozygous strains of rats show a wide range of plasma ACE values, suggesting the existence of various alleles responsible for these different levels.
We further analyzed two strains, the BN and the Lou strains, which exhibit the most contrasting values of plasma ACE. Phenotype analysis in founders, as well as in F1 and F2 progeny from a cross between these two strains, showed that the degree of genetic determination is responsible for nearly all phenotypic variance of plasma ACE. The use, in F1 and F2 progeny, of an ACE gene microsatellite, which was found to be polymorphic between the two strains, showed that the ACE locus itself explains 80% of the degree of genetic determination of plasma ACE. These results confirm previous results obtained in an F2 intercross population between the stroke-prone spontaneously hypertensive rats and the normotensive Wistar-Kyoto strain. Thus, the observation made in normotensive rats mimics what is observed in humans, since in this latter case, a polymorphism of the ACE gene also explains a major part of genetic variance of the plasma ACE level.

To identify the physiological mechanisms explaining the genetic modulation of plasma ACE values, we measured the ACE mRNA content and the ACE activity in lungs from F2 rats. These measurements clearly showed that both the mRNA and enzyme levels, which are mainly endothelial in this tissue, were related to the ACE locus polymorphism and suggested that the ACE gene expression was controlled at the transcriptional level. Run-on experiments, performed on nuclei isolated from lungs of F2 rats bearing different alleles of the ACE polymorphism, showed that the ACE transcription rate was nearly twofold higher in F2 rats homozygous for the B allele than in F2 rats homozygous for the L allele. The results obtained with tissular ACE correlated well with plasma differences between the two strains, but we cannot exclude additional mechanisms acting on plasma ACE values. It thus appears that the constitutive endothelial expression of ACE in vivo is controlled at the transcriptional level by a polymorphism likely located on the ACE gene. The sequencing of 1200 nucleotides of the 5′ flanking region of the ACE gene in the two strains did not reveal significant differences, which could explain the different rates of transcription (data not shown). Therefore, these results raise interesting questions, as in humans, on the molecular mechanism of regulation of ACE gene expression, and its variation among rat strains, since other studied strains have intermediate plasma values.

The interaction between the ACE gene expression polymorphism and inducibility of the ACE gene in different cell types of the vascular wall was therefore studied in vitro. Analysis of covariance showed that ACE genotype influenced dexamethasone-induced ACE activity in the three vascular cell types in a different manner, by modifying either the constitutive ACE expression, as in ECs, or the slope of the inducibility, as in SMCs and fibroblasts.

In humans, the variation of the ACE concentration associated with the I/D polymorphism has been observed in plasma, T lymphocytes, and the heart, even if the relationship was weaker in tissues than in plasma, which was mainly due to technical reasons. In the rat, the relationship between ACE genotype and phenotype was similarly observed in plasma, tissues, and cultured vascular cells. Therefore, the ACE gene polymorphism in rats offers the possibility to test in vivo physiological effects of contrasted levels of ACE gene expression in response to various levels of vasoactive peptidic substrates of ACE.

Despite a striking difference in plasma ACE activity between the two strains, Lou and BN rats are both normotensive. It has been demonstrated that high levels of plasma ACE such as those found in some human pathologies are not associated with high BP. Several association studies in humans using the I/D marker of ACE did not show any significant relationship with BP. However, occasional positive associations between the DD genotype and hypertension have been reported. As in the plasma, there is a twofold difference in ACE activity in the lung between the BN and the Lou strains. The

<table>
<thead>
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<th>TABLE 4. Plasma Renin-Angiotensin System Components*</th>
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<tr>
<td><strong>PRA (ng Al/mL · h⁻¹)</strong></td>
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<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Lou</td>
</tr>
<tr>
<td>BN</td>
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<td>NS</td>
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PRA indicates plasma renin activity; AI, angiotensin I; and NS, not significant.

*Results are expressed as mean±SEM.

Figure 6. Neointima proliferation 2 weeks after balloon injury. A, The intimal proliferation was greater in the BB F2 (-datepicker) than in the LL F2 (datepicker) progeny, whereas the medial thickness did not differ between the two groups. B, No differences were observed in the luminal and medial perimeters. C, The neointima/media ratio was significantly higher in the BB F2 rats than in the LL F2 rats. D, Cell density in the neointima was significantly higher in the BB F2 rats than in the LL F2 rats, whereas no differences were observed in the media.
pulmonary vascular bed is well known to be the primary site for the “in vivo” conversion of plasma angiotensin I, and it has been suggested that the majority of plasma ACE is released by the pulmonary ECs. On the other hand, there are no significant differences in the plasma renin activity and the plasma angiotensinogen, angiotensin I, and angiotensin II concentrations between the two strains. Because of its high expression in the plasma–endothelium compartment, ACE is not a limiting step in the generation of angiotensin II or in the degradation of bradykinin in the plasma. Therefore, the genetically determined modulation of ACE expression did not interfere with the pressor or the depressor responses to intravenous vasoactive peptides, the conversion or degradation of which is ACE dependent, such as angiotensin I or bradykinin. In humans the implications of the I/D genotype for the response to angiotensin I are controversial. Ueda and coworkers showed an enhanced pressor response to angiotensin I in normotensive men with the DD genotype. On the other hand, our results in rats are in accordance with the results of Lachuré et al. in normotensive rabbits. This effect was dose dependent, but the doses of angiotensin II and ACE inhibition restored the proliferative response to angiotensin II production because simultaneous infusion of angiotensin II and ACE inhibition interfered with the pressor or the depressor responses to intravenous vasoactive peptides, the conversion or degradation of which is ACE dependent, such as angiotensin I or bradykinin. In humans the implications of the I/D genotype for the response to angiotensin I are controversial. Ueda and coworkers showed an enhanced pressor response to angiotensin I in normotensive men with the DD genotype. On the other hand, our results in rats are in accordance with the results of Lachuré and coworkers in humans, which suggest that plasma ACE levels do not modify the rate of angiotensin II generation in plasma.

Beyond their functional vasomotor effects, vasoactive peptides and ACE are also involved in arterial wall postinjury remodeling. Several experimental studies have implicated the role of the renin–angiotensin system in the vascular response to endothelial injury. Administration of ACE inhibitors in rat, guinea pig, and rabbit inhibits neointimal development after arterial balloon denudation. This effect was dose dependent, but the doses of ACE inhibitors that were effective in suppressing neointima formation appeared to be higher than those used to lower BP. This discrepancy suggests a role of the local renin–angiotensin system in the pathogenesis of this model. The protective effect of ACE inhibitors appeared to be mediated by the inhibition of angiotensin II production because simultaneous infusion of angiotensin II and ACE inhibition restored the proliferative response to vascular injury and because specific angiotensin II receptor antagonism can partially inhibit neointima formation. In vitro angiotensin II has been shown to stimulate vascular smooth muscle growth and migration. Moreover, angiotensin II can be synthesized by local ACE. In contrast to plasma and endothelium, the level of basal ACE expression is low in vascular SMCs and mainly inducible. ACE overexpression in SMCs and fibroblasts may be one of the determining factors involving vasoactive peptides in the structural responses to vascular wall injury. The homozygous F2 progeny was used to test the relationship between ACE genotype and vascular remodeling in response to balloononing. The Lou and BN are both inbred rat strains. Genomic (data not shown) and phenotypic differences are numerous between these two strains. In the F2 progeny the entire genome of the two strains is mixed at random. Thus, differences observed in the balloon injury response, due to genetic differences other than the ACE gene, were minimized. Moreover, response to carotid injury is multifactorial, dependent in part on BP and on arterial wall structure (a close relation exists between body weight and arterial wall thickness). In the parental strains, significant differences were observed in BP and in body and heart weights, whereas in the ACE homozygous F2 progeny no significant differences were observed. Our data showed a significant increase in intimal proliferation, due probably to an increase in SMC proliferation in the F2 progeny with high ACE expression compared to those with low ACE expression. These data suggest that genetically determined ACE expression may influence the vascular wall response to injury, as proposed by Morishita and coworkers’ using ACE gene somatic transfer in the vascular wall.

Results of epidemiological studies in humans are contradictory. Many studies have shown that the ACE I/D polymorphism in humans is not associated with restenosis after balloon angioplasty. Moreover, two randomized trials (MERCIATOR and MARCIATOR) have failed to demonstrate any beneficial effect of ACE inhibition on the occurrence of angiographic restenosis after balloon angioplasty. Nevertheless, experimental and clinical studies have suggested that the contribution of neointimal hyperplasia to restenosis after balloon angioplasty is relatively limited and that lumen narrowing is in fact related primarily to vessel remodeling. Amant and coworkers recently demonstrated that the D allele of the ACE gene is associated with a greater late luminal loss after intracoronary stent implantation. In this case the stent prevents the remodeling process, and restenosis is primarily a consequence of neointimal hyperplasia within the stent. In accordance with this, a high expression of ACE was associated with a statistically significant increase in common carotid artery intima-media thickening, and an association between DD genotype and the extent of common carotid artery intima-media thickening has been reported. Those clinical data are concordant with our experimental results.

In conclusion, we observed a polymorphism in plasma ACE levels in different strains of rats similar to that observed in humans. We have demonstrated that the ACE gene is responsible for the major part of the genetic variance of plasma ACE levels and that the ACE gene is genetically regulated at the transcription level. In the experimental model of vascular injury by balloononing, the higher levels of ACE gene expression and inducibility are associated with a different neointima response. The ACE genotype appears to determine the ACE gene promoter activity in response to environmental stimuli. Nevertheless, such interactions between ACE genotype and cardiovascular responses to injury need to be confirmed using experimental models in congenic rats rather than in the F2 generation, where the genetic background remains heterogeneous between subjects. These data emphasize the importance of further studies trying to explain how the ACE gene polymorphism interacts with cardiovascular pathologies and to define more exactly its molecular basis in rats as well as humans.

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Angiotensin I-Converting Enzyme Genotype Influences Arterial Response to Injury in Normotensive Rats

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