Angiotensin-Converting Enzyme Gene Mutations, Blood Pressures, and Cardiovascular Homeostasis

John H Krege, Hyung-Suk Kim, Jeffrey S Moyer, J Charles Jennette, Li Peng, Sylvia K Hiller, Oliver Smithies

Abstract A common polymorphism of the angiotensin-converting enzyme (ACE) gene (ACE in humans, Ace in mice) is associated with differences in circulating ACE levels that may confer a differential risk for cardiovascular diseases. To study the effects of genetically determined changes in Ace gene function within a defined genetic and environmental background, we have studied mice having one, two, or three functional copies of the Ace gene at its normal chromosomal location. ACE activities in the serum increased progressively from 62% of normal in the one-copy animals to 144% of normal in the three-copy animals \((P < 10^{-15}, n = 132)\). The blood pressures of the mice having from one to three copies of the Ace gene did not differ significantly, but the heart rates, heart weights, and renal tubulointerstitial volumes decreased significantly with increasing Ace copy number. The level of kidney renn mRNA in the one-copy mice was increased to 129 ± 5% relative to that of the normal two-copy mice \((100 ± 4\%, P = 0.1, n = 16)\). We conclude that significant homeostatic adaptations successfully normalize the blood pressures of mice that have quantitative changes in Ace gene function. Our results suggest only that quantitative changes in expression of the Ace gene will observably affect blood pressures when accompanied by additional environmental or genetic factors that together with Ace exceed the capacity of the homeostatic mechanisms. (Hypertension. 1997;29(part 2):150-157.)

Key Words • genetics • heart rate • renn • mice

Blood pressure is a complex trait determined by an array of interlocking homeostatic systems with feedbacks that maintain homeostasis in the face of widely varying environmental factors. In some individuals, however, a pathological interaction of genetic and environmental factors results in hypertension, conferring increased risk for myocardial infarction, stroke, and renal failure.

A region of chromosome 10 was found to influence blood pressures in an F2 cross between stroke-prone spontaneously hypertensive rats and normotensive Wistar-Kyoto rats. This chromosomal region included the gene encoding ACE, a major enzyme of the renn-angiotensin and kallikrein-kinin systems. ACE catalyzes both the conversion of angiotensin I to the vasoconstricting peptide angiotensin II and also the inactivation of the vasodilating peptide bradykinin. Inhibitors of this enzyme are commonly used to treat hypertension. Thus, the ACE gene is a candidate gene for essential hypertension in humans.

To study the genetics of hypertension, we have been investigating in mice the systemic effects of induced mutations in candidate genes. We have insertionally inactivated the Ace gene and have observed that homozygous mutant mice that lack ACE have blood pressures reduced by ≈ 34 mm Hg compared with wild type.

These data establish that the Ace gene is essential for maintenance of normal blood pressures. A common polymorphism of the human ACE gene is associated with quantitative differences in circulating ACE activities. To study the overall systemic effects of quantitative genetic changes of this type, we have described a strategy using gene targeting that involves the generation of one strain of mice carrying an inactivation of the target gene and a second strain carrying a duplication of the target gene at its normal chromosomal location. Compared with normal mice that have two functional target genes, mice heterozygous for the inactivation have only one functional target gene and consequently a reduction in the overall level of target gene function, mice heterozygous for the duplication have three functional target genes and consequently an increase in the overall level of target gene function. Thus, studies in mice having one, two, and three copies of the target gene can be performed to determine the relationship between the level of target gene function and phenotypes of interest. We have previously used this strategy to show that quantitative changes in the level of function of the angiotensinogen gene directly influence blood pressures in mice. We here apply this approach to study the cardiovascular effects of quantitatively varying the level of function of the Ace gene.

Methods

Generation of Mice

To duplicate the Ace gene (Fig 1A), DNA regions upstream and downstream of the Ace gene were cloned from a λ phage library of Sau3A partially digested strain 129 DNA. An 11-kilobase (kb) EcoRI fragment was subcloned from a phage plaque that hybridized to two adjoining probes from the 5' end of the Ace gene (Table 1, probes 1 and 2). This fragment overlaps at its 3' end our previously cloned BamHI fragment. A 7-kb EcoRI
translated regions in Ace cDNAs, a shorter 3' untranslated region indicated by the bars above the targeted locus.

stem cells (sample 4) digested with BgII or double digested with BgII and the enzymes shown and hybridized to the probe in-

of the Ace gene separated by the neomycin-resistance gene. D, the targeting construct is shown by the large X. The gap (a^20 kb) is filled in by chromosomal information resulting in two copies of the Ace gene. The targeting construct was linearized with XhoI and ele-
troporated into the BK4 subline of the 14T2a strain 129 embryonic DNA from correctly targeted embryonic stem cells gives: after BgII digestion, an endogenous 8.4-kb band and an additional 5.4-kb band that has half the intensity of the 8.4-kb band; after BglII/HindIII double digestion, an endogenous 5.2-kb band and an additional 4.6-kb band that has half the intensity of the 5.2-kb band; after BglII/BamHI double digestion, an endogenous 3.5-kb band that has one-and-a-half times normal intensity; after EcoRI/BglII double digestion, the endogenous 4-kb band and an additional 3.8-kb band that has an intensity one half that of the 4-kb band. Embryonic stem cells judged to be correctly targeted by PCR and Southern blot analysis were in-
terected into C57BL/6J blastocysts. One male chimera was gener-
ated which when mated to C57BL/6J females transmitted the strain 129 genome to its wild type and mutant F1 offspring.

Environment
Mice were housed two to four per microisolator cage and were maintained on a 12-hour light/dark cycle. They were fed standard Agway R-M-H3500 autoclaved chow. Experiments were ap-
proved by the Institutional Animal Care and Use Committee at the University of North Carolina and are consistent with the Guide for the Care and Use of Laboratory Animals published by the National Academy Press (revised 1996).

Blood Pressure Evaluation
A major aim of our study was to compare the baseline blood pressures and heart rates of mice having genetically determined changes in ACE activities. Tail-cuff blood pressures and heart rates were determined in blinded fashion using a BP2000 automated, computerized, noninvasive system (Visitech) as previously described.10 Mice were aged 84 to 211 days, with group means ranging from 116 to 122 days.

We have previously found that our tail-cuff method for measuring blood pressures in mice is reproducible and provides re-

TABLE 1. Probes Used For Cloning 5' and 3' Sequences of the Ace Gene

<table>
<thead>
<tr>
<th>Probe</th>
<th>Genomic Sequences</th>
<th>Left Primer*</th>
<th>Right Primer*</th>
<th>Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Promoter and exon 1</td>
<td>TGGGACCCGCTGGAGGCCTCTGAG</td>
<td>CACTGGCAGCTGGTATAGC</td>
<td>1200</td>
</tr>
<tr>
<td>2</td>
<td>Exon 1 to exon 2</td>
<td>TATGGCTGCGCCGCCCTGCTCGTTAG</td>
<td>CTGGAAACTCTCGGCGTGAC</td>
<td>429</td>
</tr>
<tr>
<td>3</td>
<td>Last two exons</td>
<td>TCTTCTGGGCTGGGCTACGTGAGA</td>
<td>AGGGCTGATGCGACTCCA</td>
<td>480</td>
</tr>
<tr>
<td>4</td>
<td>Last exon and 3' untranslated</td>
<td>CAGCGCTGCTGAGACACAGAGG</td>
<td>CGTGAGTTGGTACAGCTGGTA</td>
<td>277</td>
</tr>
<tr>
<td>5</td>
<td>3' untranslated of ACE.5</td>
<td>AACAAGGAGCTGGCTGTCCTCTT</td>
<td>CTACTGGGCGCTGGTACAGGAA</td>
<td>466</td>
</tr>
<tr>
<td>6</td>
<td>3' untranslated of ACE.11</td>
<td>AAGCGGGGTTGCTTCCCGGGGGTAGA</td>
<td>CTGGATGTCCTGATGTCGGG</td>
<td>604</td>
</tr>
</tbody>
</table>

*Primer sequences are given in 5' to 3' orientation and were selected from sequences published by Bernstein and colleagues.
Euthanasia and Processing of Animals

To determine the effects of Ace genotype on ACE activities, hematocrit, kidney histology and structure, and organ weights, we collected serum and tissues from one-, two-, and three-copy male and female mice using an inhaled anesthetic composed of 2.5% isoflurane (Baird). Male and female mice aged 104 to 228 days with group mean body mass ranging from 142 to 152 days were anesthetized with 2.5% isoflurane and weighed. Blood was collected from the retro-orbital sinus into three nonheparinized microhematocrit tubes. Hematocrits were determined in triplicate, and serum was frozen at −70°C. Hearts, lungs, the left kidney, and testes or ovaries were harvested, weighed, and rapidly frozen in liquid nitrogen and stored at −70°C. The right kidney was fixed in 4% neutral buffered formalin for histology.

ACE Activities

To determine the effect of Ace gene copy number on Ace gene function, we measured ACE activities in serum and in lung. Serum ACE activities were measured in blinded fashion using scaled-down colorimetric methods with a commercially available kit (Sigma Chemical Co). For lung ACE activities, one lung was homogenized in 4 mL of 0.05 M Tris pH 7.5, 0.3 M NaCl and centrifuged at 6000 rpm for 20 minutes. The supernatant was tested for ACE activity in blinded fashion using the same kit.

Kidney Histology and Morphometry

Because of the central importance of the kidney in blood pressure control, we evaluated in blinded fashion the histology and some morphometric parameters of kidney structure of hematocrit and eosin-stained sections of kidneys from one-, two-, and three-copy male and female age-matched mice. For the morphometric analysis, kidney images were captured and analyzed using NIH Images software. Average cortical width was defined as the average of the cortical width measured at five uniform locations. The average glomerular cortical area subtending a single glomerulus, was defined as the average cortical area minus the total glomerular area divided by the number of glomeruli present within the six cortical fields.

Evaluation of Kidney Renin mRNA

To study to what extent the renin gene compensates for changes in Ace gene function, we quantitated in blinded fashion the steady-state level of renin gene transcripts in one- and two-copy genetically uniform F1 male and female mice using an RNase protection assay. Total kidney RNA was isolated using guanidium/isoisothiocyanate (1). Riboprobes labeled with 32P were transcribed from a 290-bp fragment from exon 9 of the mouse renin gene (2). The riboprobe was hybridized in solution with total RNA from the kidney specimens. After RNase treatment, the protected fragments were precipitated with trichloroacetic acid, filtered through a glass filter, and 32P cpm determined by scintillation. To estimate the concentration of renin mRNA, a standard curve was constructed by hybridizing the 32P-labeled renin probe with known quantities of renin cRNA that had been transcribed in vitro. Renin mRNA levels are expressed as picograms of renin mRNA per microgram of total kidney RNA.

Statistical Analysis

Data are expressed as mean±SEM. For all phenotypes, we performed two-factor ANOVA with sex and genotype as factors. We used regression analysis to determine the slopes of dose-response relationships.

Results

Generation of Animals

To generate mice having a duplication of the Ace gene, the targeting construct shown in Fig 1 was electroporated into embryonic stem cells. Of 94 cell lines doubly resistant to G418 and ganciclovir, 5 were correctly targeted as judged by PCR and Southern blot analysis. Injection of one cell line into strain C57BL/6J blastocysts reimplanted into CD1 foster mothers gave a chimera that transmitted the strain 129/Ola embryonic stem cell genome to its F1 wild-type and mutant offspring.

The generation of mice having an insertional disruption of exon 14 of the Ace gene has been described previously (3). Additional mice having reduced Ace gene function caused by a mutation that replaces most of exon 10 with the neomycin-resistance gene were also generated (J. R. Hagaman et al., unpublished data, 1996). Mice having the exon 10 disruption were not significantly different from mice having the exon 14 disruption in ACE activities or in any of the phenotypes reported herein, and the results for one-copy mice include the pooled results from the exon 10 and exon 14 mutant mice.

To generate the experimental animals, F1 mice (strains 129/OlaxC57BL/6J) heterozygous for the different mutations were intercrossed to generate F2 mice having one, two, three, or four copies of the Ace gene. Genotyping for the one-copy mice was by Bam HI digestion of tail DNA and Southern blot analysis using probes from exon 12 or the testes-specific exon as previously described (3). Several diagnostic tests were performed to genotype the duplication mice. Southern blot analysis of genomic tail DNA after BglII digestion and hybridization with a probe to the neomycin-resistance gene gives a 5.4-kb band for mice having at least one duplicated Ace locus. Digestion of tail DNA with BglII and hybridization to probe 4 (Table 1) gives an endogenous 8.4-kb band for mice of all genotypes and a 5.4-kb band in mice having three and four copies of the Ace gene. Several diagnostic tests were performed to determine the number of copies of the Ace gene; the intensity ratios of the 8.4:5.4-kb bands are 2.1 in three-copy mice and 1.1 in four-copy mice. Because of the difficulties associated with band intensity determinations in Southern blots, an additional test was used to distinguish three- from four-copy mice: the marker D11Mit258 (12) is linked to the Ace gene, and its length distinguishes C57BL/6 chromosomes (≈130 bp) from 129 chromosomes (≈166 bp). The presence of both bands indicates three-copy mice, while the presence of only the 166-bp band indicates four-copy mice. The primer sequences for D11Mit258 are left primer, 5′-AACACGAGATAAAACCGG3′, and right primer, 5′-TGTTGAACACTCTCAGAAGGC3′. Thirty-six mice that were neomycin-resistance gene–positive were tested by both the Southern blot intensity method and by the linkage method; these diagnostic tests gave identical results in all cases.

For the present work, we primarily studied one-, two-, and three-copy F2 mice respectively having reduced, normal, and increased Ace gene function. Genetically, one- and three-copy F2 mice differ systematically only in inheriting either the strain 129–disrupted Ace gene or the
strain 129–duplicated Ace gene. In the study of mRNA levels, we used F1 animals that are genetically identical except for being either wild-type or heterozygous mutant for the exon 14 Ace gene activation.

Mice of all genotypes appeared outwardly healthy

**ACE Activities**

To determine the effects of Ace genotype on Ace gene function, we measured serum and lung ACE enzymatic activities in one-, two-, and three-copy male and female mice. Serum ACE activities (Fig. 2) were strongly influenced by both Ace genotype ($P<10^{-15}$) and sex ($P<10^{-15}$). Expressed as a percentage of the mean of like-sexed wild-type mice, the ACE activities of the pooled male and female mice were one copy, $62\pm2\%$ (n=49), two copy, $100\pm8\%$ (n=46), and three copy, $144\pm4\%$ (n=33).

Lung ACE activities (units/milligram protein) were similar to serum ACE activities. Lung ACE activity was strongly influenced by both Ace genotype ($P<10^{-5}$) and sex ($P=0.01$). The lung ACE activities of the pooled male and female mice were one copy, $60\pm7\%$ (n=29), two copy, $100\pm8\%$ (n=31), and three copy, $138\pm16\%$ (n=17).

**Blood Pressures, Heart Rates, and Hematocrits**

To determine whether genetic changes in ACE affect resting blood pressures or heart rates, we studied 154 male and female mice having from one to three copies of the Ace gene. Blood pressures (Fig 3A) were not significantly affected by Ace gene copy number ($P=0.20$), sex ($P=0.38$), or the interaction of copy number and sex ($P=0.69$). In the same 154 mice, heart rates (Fig 3B) were significantly affected by Ace gene copy number ($P=0.001$) but not by sex ($P=0.52$) or by the interaction of copy number with sex ($P=0.20$). The slope of this dose-response relationship was $-16\pm5$ bpm per gene copy number ($P=0.001$), which represents an $\approx2.3\%$ decrease in heart rate for a unit increase in Ace gene copy number.

As an indicator of vascular volumes (assuming equal total red blood cell volumes), we determined the hematocrits of 104 mice; this phenotype was not significantly affected by Ace gene copy number ($P=0.25$), although as expected, male mice demonstrated significantly higher weights than female mice ($P<0.001$). There was no significant interaction of genotype and sex ($P=0.52$).

Heart weights (mg/g body weight) of 105 mice were significantly affected by Ace gene copy number ($P=0.008$) but not by sex ($P=0.16$) or the interaction of sex and copy number ($P=0.72$). The slope of the dose-response relationship was $-0.26\pm0.09$ mg/g body weight per Ace gene copy ($P=0.003$), which represents an $\approx5\%$ decrease in heart weight for a unit increase in Ace gene copy number.

Kidney, lung, and gonad weights were strongly affected by sex but were not significantly influenced by Ace gene copy number or the interaction of sex and copy number.

**Body and Organ Weights**

We determined whether quantitative changes in Ace gene function influence whole-body, heart, kidney, lung, or gonad weights by weighing some of the animals and their organs (Table 2).

In 106 F2 mice, body weights were not significantly affected by Ace gene copy number ($P=0.25$), although as expected, male mice demonstrated significantly higher weights than female mice ($P<0.001$). There was no significant interaction of genotype and sex ($P=0.52$).

Heart weights (mg/g body weight) of 105 mice were significantly affected by Ace gene copy number ($P=0.008$) but not by sex ($P=0.16$) or the interaction of sex and copy number ($P=0.72$). The slope of the dose-response relationship was $-0.26\pm0.09$ mg/g body weight per Ace gene copy ($P=0.003$), which represents an $\approx5\%$ decrease in heart weight for a unit increase in Ace gene copy number.

Kidney, lung, and gonad weights were strongly affected by sex but were not significantly influenced by Ace gene copy number or the interaction of sex and copy number.

**Kidney Histology and Morphometry**

We determined whether quantitative changes in Ace gene function affect kidney structure. By light microscopy, increasing Ace gene copy number appeared to be associated with decreasing tubulointerstitial tissue per glomerulus. In mice of all genotypes, the tubulointerstitial compartment was composed almost entirely of tubules and was without inflammation, interstitial fibrosis, or other overt abnormalities. Arteries and arterioles appeared structurally normal and did not show sclerotic or proliferative changes.
TABLE 2. Body and Organ Weights of Mice Having One, Two, or Three Copies of the Ace Gene

<table>
<thead>
<tr>
<th>Copy No.</th>
<th>Body weights, g</th>
<th>Heart weights, mg/g</th>
<th>Kidney weights, mg/g</th>
<th>Lung weights, mg/g</th>
<th>Gonad weights, mg/g</th>
<th>Body weight, mg/g</th>
<th>Heart weight, mg/g</th>
<th>Kidney weight, mg/g</th>
<th>Lung weight, mg/g</th>
<th>Gonad weight, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 COPY</td>
<td>Females</td>
<td>Males</td>
<td>Copy No.</td>
<td>Sex</td>
<td>Copy No. x Sex*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>25.7±0.6</td>
<td>20.2±1.0</td>
<td>27.4±1.0</td>
<td>26.7±0.6</td>
<td>30.1±0.0</td>
<td>30.1±0.2</td>
<td>25</td>
<td>&lt;0.001</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>17</td>
<td>11</td>
<td>23</td>
<td>29</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5.16±0.17</td>
<td>4.74±0.10</td>
<td>4.82±0.14</td>
<td>5.42±0.17</td>
<td>5.03±0.13</td>
<td>4.84±0.20</td>
<td>0.008</td>
<td>16</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>17</td>
<td>11</td>
<td>23</td>
<td>28</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>6.44±0.22</td>
<td>6.01±0.17</td>
<td>6.66±0.29</td>
<td>7.66±0.15</td>
<td>7.16±0.24</td>
<td>7.48±0.31</td>
<td>0.007</td>
<td>&lt;0.001</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>13</td>
<td>12</td>
<td>9</td>
<td>17</td>
<td>24</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>7.40±0.31</td>
<td>7.14±0.16</td>
<td>7.62±0.40</td>
<td>6.27±0.19</td>
<td>6.57±0.23</td>
<td>6.10±0.28</td>
<td>1</td>
<td>&lt;0.001</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>13</td>
<td>12</td>
<td>9</td>
<td>17</td>
<td>24</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.84±0.33</td>
<td>3.38±0.26</td>
<td>2.84±0.18</td>
<td>3.02±0.21</td>
<td>4.32±0.32</td>
<td>4.24±0.55</td>
<td>1</td>
<td>&lt;0.001</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>13</td>
<td>12</td>
<td>8</td>
<td>16</td>
<td>24</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Interaction of copy number and sex
†Left kidney weighed

Our impressions of structural changes in the kidneys of mice having quantitative changes in the level of Ace gene function were confirmed by morphometric analysis (Table 3). We found that cortical width and the tubulointerstitial area per glomerulus decreased significantly with increasing Ace gene copy number. The slope of the dose-response relationships were: average cortical width, -104±36 μm/μm³×10³/Ace gene copy (P=0.006); tubulointerstitial area per glomerulus, -15±6 μm²/μm³×10³/Ace gene copy (P=0.028). These slopes represent an ~7% decrease in average cortical width and an ~10% decrease in tubulointerstitial area per glomerulus per unit increase in Ace gene copy number.

Kidney Renin mRNA Expression

To determine how the renn gene responds transcriptionally to genetically determined changes in Ace, we used an RNase protection assay to measure steady-state renn gene transcripts in the kidneys of an independent set of age-matched, genetically uniform F1 mice that had one or two copies of the Ace gene. The kidney renn mRNA levels (Fig 4) were increased to 129±5% (P=0.01) in one-copy compared with two-copy mice, 133±±6%, and were 133±±6% higher in females than in males (P=0.02). There was no significant interaction of genotype and sex (P=0.46).

Results From Four-Copy Mice

Our studies were directed at studying mice having subtly decreased, normal, and subtly increased Ace gene function. For this reason, we studied one-, two-, and three-copy mice extensively. We will here give the results from a small number of female mice that were also studied during the course of our investigations that were homozygous for the Ace gene duplication and so had four functional Ace genes.

The four-copy mice appeared outwardly normal. Their serum ACE activities were 356±32 U/mL (n=3) or ~31% that of two-copy females (see Fig 2), their lung ACE activities were 45±12 U/mg protein (n=3) or ~15% that of two-copy females. Their blood pressures were 114±9 mm Hg (n=4) and heart rates were 648±14 bpm (n=4); thus, although their blood pressures were similar to those of the one- and three-copy females, their heart rates continued the significant trend of decreasing heart rate with increasing Ace gene copy number (see Fig 3). Their hematocrits were 0.51±0.01 (n=3), heart weights were 4.99±0.46 mg/g body weight (n=3), kidney weights were 1.4±0.22 mg/g body weight (n=3), lung weights were 7.6±0.31 mg/g body weight (n=3), and ovary weights were 3.07±0.48 mg/g body weight (n=3). The heart rate results in these four-copy females do not show continued reduction in heart weight with increasing Ace gene copy number as was present in the one- to three-copy mice (see Table 2). Results of the kidney morphometric analysis of four-copy mice included: average cortical width, 1312±90 μm, average glomerular area, 2.33±0.25 μm²/μm³×10³, and tubulointerstitial space per glomerulus, 106±±6 μm²/μm³×10³. The kidney morphometric results in four-copy mice showed continuance of the significant trends present in the one- to three-copy mice (see Table 3) of decreasing average cortical width and tubulointerstitial area per glomerulus with increasing Ace gene copy number.

Overall, these results from four-copy mice support the trends established in the one- to three-copy mice of increasing serum and lung ACE activities and decreasing heart rate, average renal cortical width, and tubulointerstitial space per glomerulus with increasing Ace gene copy.

TABLE 3. Morphometric Analysis of Kidneys in Mice Having One, Two, or Three Copies of the Ace Gene

<table>
<thead>
<tr>
<th>Copy No.</th>
<th>Females</th>
<th>Males</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 COPY</td>
<td>1 Copy</td>
<td>2 Copy</td>
<td>3 Copy</td>
</tr>
<tr>
<td>10</td>
<td>1010±97</td>
<td>1430±29</td>
<td>1400±31</td>
</tr>
<tr>
<td>10</td>
<td>3.77±0.38</td>
<td>3.23±0.29</td>
<td>3.11±0.11</td>
</tr>
<tr>
<td>10</td>
<td>140±12</td>
<td>138±9</td>
<td>114±4</td>
</tr>
</tbody>
</table>

*Interaction of copy number and sex
†Defined for each animal as total glomerular area/number of glomeruli present
††Defined for each animal as (Total Cortical Area-Total Glomerular Area)/Number of Glomeruli Present

Downloaded from http://hyper.ahajournals.org/ by guest on January 9, 2018
number. The significant trend of decreasing heart weights with increasing Ace gene copy number did not continue in these four-copy females.

**Discussion**

A common polymorphism of the human ACE gene is associated with quantitative differences in the level of ACE gene function and the circulating ACE levels. This polymorphism has been hypothesized to influence the risk for cardiovascular diseases, including hypertension, cardiac hypertrophy, and myocardial infarction, although evidence for these hypotheses has varied in different studies. To directly study the effect of quantitative changes in Ace gene function within a defined genetic and environmental background, we have studied the blood pressures and related phenotypes of mice having from one to three copies of the Ace gene at its normal chromosomal location.

In carrying out this study, we generated animals having reduced, normal, or increased Ace gene function. For the study of mice having reduced Ace gene function, we used offspring of previously reported animals having a disruption of exon 14 within the 3′ domain of the Ace gene, and we also generated additional animals having a disruption of exon 10 within the 5′ domain of the Ace gene (J.R. Hagaman et al, unpublished data, 1996); ACE activities and cardiovascular phenotypes of these two strains of one-copy mice were indistinguishable. To study the effects of increased Ace gene function, we generated animals having a duplication of the Ace gene at its normal chromosomal location. By breeding mice carrying these three mutations, we obtained mice heterozygous for the disruption (they have one functional Ace gene), mice homozygous wild type (two functional Ace genes), and mice heterozygous for the duplication (three functional Ace genes)

The relationship between Ace gene copy number and serum ACE activities was essentially linear and dose-dependent, allowing us to study the effects on cardiovascular phenotypes of graded change in Ace gene function from ≈62% of normal to ≈144% of normal. Genetically, mice having the disruption or duplication of Ace differ systematically only at the Ace locus.

Examination of the tail-cuff pressures of the one-through three-copy mice shows that the level of Ace gene function does not directly cause any significant changes in the blood pressures of F2 mice (strain 129×B6), which have an otherwise normal genetic background. It is important to note that our assessment of the effects of Ace genotype on blood pressures was within the environment of the tail-cuff system, which includes restraint and heating. Within other environments, it is possible that different results will be obtained. Most importantly, the future availability of blood pressure telemetry systems for the mouse will allow assessment of resting blood pressures during the day and during the night in animals that have fully recovered from surgery. However, our present results are in agreement with the weight of the evidence from a variety of other studies. In mice, Esther et al demonstrated no significant differences between the blood pressures of mice wild-type or heterozygous for a loss of function mutation similar to our Ace disruption mutations. We have also recently completed a study of the effects of Ace genotype in genetically uniform F1 mice heterozygous for a disruption of the Apoe gene and fed a high-fat diet; within this (different) genetic and environmental background, the blood pressures of mice wild-type or heterozygous for the Ace gene disruption were also not significantly different.

In addition, the original finding of linkage of the genetic region containing the rat ACE gene with increased blood pressure in the stroke-prone spontaneously hypertensive rat may not be due to the ACE gene itself, since further linkage studies have indicated that inheritance of different alleles of the rat ACE gene affect plasma ACE activity but do not influence blood pressure. In humans, association of the ACE I/D polymorphism with differences in blood pressure has been observed in some but not other case-control association studies of hypertension, but in zero of nine case-control studies primarily of atherosclerosis or myocardial infarction. Additionally, the only sib-pair linkage study of the ACE gene did not find significantly increased sharing of markers linked to the human ACE gene in siblings that shared hypertension.

Two hypotheses arise from the absence of an observable effect of Ace gene function on blood pressure in our study in which the level of Ace gene function is the only systematic variable. One hypothesis is that the quantitative level of Ace gene function is immaterial. A second possibility is that homeostatic compensations occur within these otherwise intact and normal animals that eliminate observable blood pressure differences.

To test these hypotheses, we evaluated additional cardiovascular phenotypes in the experimental mice. Heart rate and heart weight were observed to be modestly but significantly inversely proportional to Ace gene copy number. Additionally, because of the central importance of the kidney in blood pressure regulation, we studied some morphometric parameters of kidney structure and found that increasing Ace gene copy number was accompanied by significantly decreasing renal cortical widths and tubulointerstitial areas per glomerulus. Finally, we observed significantly increased kidney renin mRNA levels in mice having decreased Ace gene function.

Other studies also suggest that the level of Ace gene function significantly affects phenotypes related to blood pressure control, even though it does not appear to measurably affect resting blood pressures in normal mice. Tian et al (unpublished data, 1996) have found that compared to wild-type mice, mice heterozygous for the Ace gene disruption have indistinguishable baseline blood pressures but (1) a significantly reduced blood pressure response to injected angiotensin I, (2) a significantly increased blood pressure response to injected bradykinin, and (3) a significantly increased level of expression of renin mRNA in the kidney.

**Figure 4** Kidney renin mRNA levels in F1 (strain 129/Ola×C57BL/6J) genetically uniform mice. There were four mice in each group.
The present data show that quantitative changes in Ace gene function result in roughly dose-dependent and linear changes in serum ACE activities but do not measurably influence blood pressure. These data contrast with previous observations that similar changes in the level of function of the genes for angiotensinogen and the angiotensin type Ia receptor do measurably influence blood pressure. These experiments indicate that quantitative genetic changes in the initial substrate and final receptor of the renin-angiotensin system affect blood pressure more than quantitative genetic changes of similar magnitude in the intermediate ACE. These findings also suggest the testable hypothesis that changes in the ACE activities of Ace mutant mice are not accompanied by quantitatively similar changes in the level of production of angiotensin II in vivo at steady state, decrease (or increase) in the activity of ACE may cause accumulation (or depletion) of the immediate precursor, angiotensin I, which in part restores the normal rate of angiotensin II generation.

The absence of an effect of Ace genotype on blood pressure must be considered within the context of the intact organism having multiple systems directed at maintaining blood pressure homeostasis. Many or all of these systems may respond to or adapt to changes in the genetically determined level of ACE in a compensatory manner. In the renin-angiotensin system, we have demonstrated that genetic reduction in Ace gene function is accompanied by reciprocal increases in renin gene function in the kidney. We also observed significant changes in heart rate, heart weight, and kidney structure in mice having quantitative changes in Ace gene function and no significant imposed genetic or environmental stress. These homeostatic adaptations must either result from the direct effects of changes in ACE or represent the physiological/anatomic responses to compensatory changes in other blood pressure homeostatic systems. Our observations that changes in Ace gene function result in the induction of significant homeostatic adaptations suggest that quantitative changes in expression of the Ace gene when accompanied by additional genetic or environmental factors might measurably affect blood pressures by stressing the homeostatic machinery beyond its limits. In yeast, illustratively, genetic up- or down-modulation of five separate enzymes in the tryptophan synthesis system did not measurably affect the scored phenotype of growth rate, while simultaneous increases in two or more enzymes of the pathway resulted in a significantly increased growth rate. The likelihood that the level of Ace gene function does measurably influence blood pressures in some but not other genetic or environmental backgrounds emphasizes the need to explore both genetic and environmental variants of blood pressure controlling genes and the context in which they exist to unravel the complex determination of hypertension in heterogeneous human populations.

Acknowledgments

This work was supported by grants GM20069 and HL49277 (O.S.), and HL03470 (J.H.K.) from NIH. J.H.K. was a Physician Research Fellow and J.S.M. was a Medical Student Research Fellow supported by the Howard Hughes Medical Institute. We are grateful to the W.M. Keck Foundation for a grant to the University of North Carolina to support work with animal models. We thank Eric Bachman, Denise Lee, Goo-Taeg Oh, Gloria O’Neal, and Laura Langenbach Tuning for their excellent help.

References


35. Shiu SY, Langford KG, Martel BM, Bernstein KE. Genomic DNA 5' to the mouse and human angiotensin-converting enzyme genes contains two distinct regions of conserved sequence. *Biochem Biophys Res Commun* 1990,167 1128-1133
Angiotensin-Converting Enzyme Gene Mutations, Blood Pressures, and Cardiovascular Homeostasis

John H. Krege, Hyung-Suk Kim, Jeffrey S. Moyer, J. Charles Jennette, Li Peng, Sylvia K. Hiller and Oliver Smithies

Hypertension. 1997;29:150-157
doi: 10.1161/01.HYP.29.1.150

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1997 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/29/1/150

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/