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Appropriate Regulation of Renin and Blood Pressure in 45-kb Human Renin/Human Angiotensinogen Transgenic Mice

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Abstract—The renin-angiotensin system is normally subject to servo control mechanisms that suppress plasma renin levels in response to increased blood pressure and increase plasma renin levels when blood pressure falls. In most species, renin is rate limiting, and angiotensinogen circulates at a concentration close to the K_m , so varying the concentration of either can affect the rate of angiotensin formation. However, only the plasma renin level responds to changes in blood pressure and sodium balance to maintain blood pressure homeostasis. Therefore, the high plasma human renin levels and the hypertension of mice and rats containing both human renin and angiotensinogen transgenes indicate inappropriate regulation of renin and blood pressure. These anomalies led us to develop new lines of transgenic mice with a longer human renin gene fragment (45 kb) than earlier lines (13 to 15 kb). Unlike their predecessors, the 45-kb *hREN* mice secrete human renin only from the kidneys, and both the human and mouse renins respond appropriately to physiological stimuli. To determine whether blood pressure is also regulated appropriately, we crossed these new 45-kb *hREN* mice with mice containing the human angiotensinogen gene. All doubly transgenic mice were normotensive like their singly transgenic and nontransgenic littermates. Moreover, among doubly transgenic mice, both human and mouse plasma renin concentrations were suppressed relative to the singly transgenic 45-kb *hREN* mice. These findings demonstrate the importance of appropriate cell and tissue specificity of gene expression in constructing transgenic models and affirm the pivotal role played by renal renin secretion in blood pressure control. (*Hypertension*. 1999;33[part II]:318-322.)

Key Words: renin ■ angiotensinogen ■ kidney ■ mice ■ gene expression ■ renin-angiotensin system

Circulating renin is synthesized in the renal juxtaglomerular cells and secreted by the kidneys in response to reduced blood pressure and sodium load and to increased β -adrenergic stimulation. The ability of the kidney to direct renin secretion appropriately in response to these physiological effectors depends on both the biochemical characteristics of the juxtaglomerular cells, such as their complement of receptors and their unique ability to process prorenin, and their anatomic localization within the kidney. Therefore, for renin to serve its normal physiological function, it must be appropriately expressed in the renal juxtaglomerular cells.

Transgenic mice provide the opportunity to study human renin *hREN* gene expression in its native cellular environment. Moreover, the species specificity of the reaction between renin and its substrate prevents human renin from exerting a physiological effect unless human angiotensinogen (AGT) is also present. In recent years, a number of transgenic mice and rats expressing both *REN* and *hAGT* genes have been created (Table 1). In lines in which plasma levels of human renin and human AGT were both elevated, plasma renin activities increased, and the animals became hypertensive.¹⁻³

But are the elevated blood pressure and high plasma renin levels in these animals physiologically appropriate?

Normally, renin is released from the kidney when blood pressure falls or when the sodium chloride load sensed by the macula densa cells of the kidney is reduced. The angiotensin (Ang) II generated by the renin constricts the vasculature and promotes sodium retention, thus reestablishing blood pressure and plasma electrolytes. In so doing, the normal servo control mechanisms suppress renin secretion that is no longer required. Thus, high plasma renin activity (PRA) in the presence of high blood pressure is abnormal, suggesting a defect in the mechanisms that regulate human renin secretion in existing transgenic models.

Although all the previously developed human renin transgenic lines synthesize human renin in renal juxtaglomerular cells, they also express the *hREN* gene at a number of unusual extrarenal sites, such as adipose, intestines, spleen, stomach, and skeletal muscle.⁴⁻⁷ We recently showed in 1 of these lines that human renin is secreted into the plasma from tissues other than the kidneys.⁷ These extrarenal sites presumably lack the mechanisms to correctly modulate renin secretion in response to physiological changes. Thus, even though human

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TABLE 1. Renin-Angiotensin System Parameters and Blood Pressure in Transgenic Rodent Models Carrying Human Renin and Angiotensinogen Genes

| Study | Species | <i>hREN</i> , kb | <i>hAGT</i> ,* kb | <i>hPRC</i> * | <i>hAGT</i> * | PRA | Δ BP |
|------------------------------|---------|------------------|-------------------|---------------|---------------|-----|-------------|
| Ganten et al ⁶ | Rat | 15 kb | 14 kb | 0.5 | 6.6 | ND | 0 |
| Fukamizu et al ² | Mouse | 15 kb | 14 kb | 50 | 8.3 | 85 | 30 |
| Merrill et al ³ | Mouse | 13 kb | 14 kb | 30 | 3.8 | 100 | 40 |
| Bohlender et al ¹ | Rat | 15 kb | 14 kb | 270 | 78 | 180 | 75 |

BP indicates blood pressure.

**hPRC* and *hPRA* are in ng Ang I \cdot mL⁻¹ \cdot h⁻¹. *hAGT* is expressed in μ g Ang I/mL.

renin secretion from the kidneys might respond appropriately,⁸ inappropriate secretion from extrarenal sites would maintain an elevated blood pressure.

We recently developed new transgenic mouse lines that contain 45-kb human renin genomic sequences⁷ with much longer 5'- and 3'-flanking DNA sequences (\approx 20 and \approx 8 kb, respectively) than previous models.^{4,5} In these mice, human renin mRNA is expressed at few, mainly normal, extrarenal sites; more importantly, circulating human renin is secreted exclusively by the kidneys. Circulating human renin in these mice also responds appropriately (in parallel with the endogenous mouse renin) to changes in dietary sodium, converting enzyme inhibition and β -adrenergic stimulation.⁹ Moreover, infusion of mouse or human AGT results in an increase in blood pressure that suppresses both human and mouse renin secretion in these animals.⁹

In the present study, we investigated the effect of making 45-kb *hREN* mice doubly transgenic for the *hAGT* gene. Mice carrying a *hAGT* transgene¹⁰ have 4 to 5 times the normal human levels of human AGT circulating in their plasma. In contrast to previous studies, we show that the doubly transgenic mice are normotensive and that their plasma levels of human and mouse renins are suppressed. This results in a constant PRA that is consistent with their normotension. These studies demonstrate the importance of correct cell- and tissue-specific renin gene expression in maintaining normal blood pressure and affirm the pivotal role played by renal renin secretion in blood pressure control.

Methods

Animals

All experiments with animals were approved by the Institutional Animal Care and Use Committee of the Weill Medical College, Cornell University.

We prepared 45-kb *hREN* transgenic mice using a 45-kb *Not I-Sal I* fragment from the P1 human renin genomic clone number 3969¹¹ as described.⁷ The original founder mice were back-crossed to C57BL6 through 4 or 5 generations. *hAGT* mice containing a 14-kb *hAGT* genomic fragment¹⁰ were obtained from Jackson Labs. Transgenic animals were identified by Southern blotting of DNA extracted from tail tips. Mice were housed under standard conditions with free access to tap water and Formulab mouse chow (No. 5008, containing 0.28% sodium).

Blood samples (\approx 200 μ L) were obtained by orbit sinus puncture and assayed for mouse and human renin, total renin, and AGT as described.⁹ However, the concentration of human substrate in the *hPRC* assay was increased 6-fold to a level close to K_m . PRA was determined by incubating 75 μ L of plasma for 3 hours at 37°C with an equal volume of buffer (pH 5.6) containing 3 mmol/L EDTA and 0.04% phenylmethylsulfonyl fluoride.¹²

Blood pressure was measured with a Visitech 2000 automated tail-cuff sphygmomanometer. Blood pressure measurements were routinely carried out between 9 AM and noon. Animals were acclimated to the tail cuff and measurement procedure for 3 days, after which consistent blood pressure measurements were obtained. Animals were routinely subjected to 5 preliminary cycles and 10 measurement cycles. Usually, >70% of cycles yielded a measurement. The SE for each set of 10 measurement cycles was typically >10% of the mean.

In a sampling of mice from all the transgenic litters generated in this study, blood pressure was also measured directly while the mice were conscious. Blood pressures were measured by tail cuff in the morning. The mice were then catheterized and allowed to recover, and direct measurements were taken in the afternoon or on the following day. There was a strong correlation between systolic blood pressure measured by tail cuff (TC) and mean arterial pressure (MAP) measured directly: MAP=5.8+0.9(TC); $r^2=0.54$; range, 90 to 120 mm Hg; n=8.

Statistical Analysis

All values are expressed as mean \pm SEM. Comparisons between mouse and human renin and AGT measurements and blood pressure and heart rate measurements for the 4 possible transgenic genotypes were tested by ANOVA. The significance of multiple comparisons was assessed by Scheffé's test. A value of $P<0.05$ was considered statistically significant.

Results

Matings were established between animals heterozygous for each transgene. In 5 separate breedings, male or female 45-kb *hREN* mice were mated with 14-kb *hAGT* mice. Among the 31 offspring, the number of each genotype was 7 *hREN/hAGT*, 6 *hREN*, 12 *hAGT*, and 6 nontransgenic mice, which did not differ significantly from the numbers expected by random assortment of alleles ($\chi^2=1.11$, $P=NS$). All animals developed normally and reached similar weights for their sex (data not shown).

Blood Pressure

Blood pressure measurements were begun at 4 weeks of age, and blood samples were drawn at 4 and 8 weeks. In all mice, regardless of their transgenic genotype, blood pressure and heart rate remained normal (Table 2).

Plasma Levels of Human and Mouse Renin and AGT

In the present study, in singly transgenic 45-kb *hREN* mice, human plasma renin concentration (PRC) averaged 98 ± 12 ng Ang I \cdot mL⁻¹ \cdot h⁻¹, and human plasma total renin concentration (TRC) averaged 156 ± 18 ng Ang I \cdot mL⁻¹ \cdot h⁻¹. In nontransgenic mice, mouse TRC averaged 1314 ± 217 ng Ang I \cdot mL⁻¹ \cdot h⁻¹, and mouse PRC averaged 850 ± 179 . Normal

TABLE 2. Blood Pressure and Heart Rate in Progeny of *hREN*×*hAGT* Transgenic Mice at 4 and 8 Weeks of Age

| Genotype | 4 w | | | | 8 w | | | |
|------------------|-------------------------------|-----------------|--------------------|---|-------------------------------|-----------------|--------------------|----|
| | Mean Systolic Pressure, mm Hg | Heart Rate, bpm | >70% Successful, % | n | Mean Systolic Pressure, mm Hg | Heart Rate, bpm | >70% Successful, % | n |
| <i>hREN/hAGT</i> | 94±6 | 634±35 | 83 | 6 | 96±4 | 662±21 | 100 | 8 |
| <i>hREN</i> - | 96±4 | 716±22 | 100 | 5 | 97±3 | 648±12 | 100 | 6 |
| -/ <i>hAGT</i> | 92±4 | 706±11 | 100 | 8 | 103±3 | 649±15 | 67 | 12 |
| -/- | 93±9 | 658±41 | 67 | 3 | 100±4 | 639±34 | 83 | 6 |

Values are mean±SEM when appropriate.

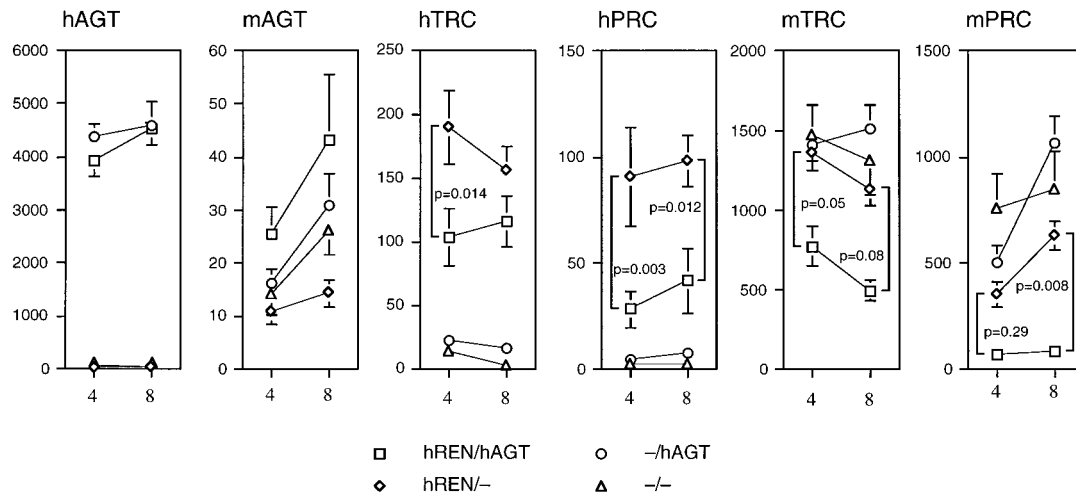
human PRC is typically ≈10 ng Ang I · mL⁻¹ · h⁻¹. The extraordinarily high mouse levels are similar to results from our earlier study and reports from other laboratories. Normal plasma levels of renin-angiotensin system components in mice and humans are reviewed elsewhere⁹ Measurements of human PRC and TRC were higher than previously reported⁹ because of the higher substrate concentration used in assays for the present study. Nevertheless, in the present study, the proportion of total renin secreted as active renin was 62% for human and 65% for mouse, which are also very similar to our previous report (57% and 63% for human and mouse renins, respectively).⁹ As described previously, mouse PRC was lower in *hREN* mice than in control mice, although these differences were not significant at either time point.

Most importantly, *hREN* mice that carried the *hAGT* gene had lower human and mouse PRC compared with singly transgenic *hREN* mice (the Figure). In the doubly transgenic mice, human PRC was suppressed 3.2-fold at 2 weeks and 2.3-fold at 4 weeks, relative to singly transgenic *hREN* mice. Mouse PRC was suppressed even further, by 5.2-fold at 1 month and 7.3-fold at 2 months. In doubly transgenic animals, plasma levels of human AGT remained relatively constant between 4 and 8 weeks, ranging from 4000 to 4500 ng Ang I · mL⁻¹ · h⁻¹, which is about 5 times the normal human level. There were no significant differences in plasma levels of human AGT between animals that carried only the

hAGT transgene and those that also carried the *hREN* transgene.

In 1 group of mice, PRA levels were determined at 8 weeks of age. PRA is the ability of plasma to generate Ang I and reflects the plasma concentration of both renin and AGT. Despite the differences in mouse and human PRC and substrate levels, PRA levels were remarkably similar in all animals, regardless of their transgenic type (range, 20 to 40 ng Ang I · mL⁻¹ · h⁻¹). This suggests that renin secretion changed in response to differences in AGT to maintain a constant rate of angiotensin formation.

Although not directly related to the major conclusions of this study, there are a number of additional noteworthy observations. First, mouse AGT levels were determined by the ability of hog renin to generate Ang I from mouse plasma. Although hog renin does not significantly cleave human AGT, the rate of Ang I generation from the plasma of animals carrying the *hAGT* transgene was 2- to 3-fold higher than animals expressing only the endogenous *mAGT* gene. Mouse AGT levels also appeared to be lower in animals expressing the *hREN* transgene. One possible explanation for this finding is that human renin may bind mouse AGT without cleaving it, thus acting as a competitor for mouse renin. Second, human TRC did not differ significantly between doubly transgenic mice and mice carrying *hREN* alone. However, mouse TRC was suppressed 1.8-fold at 1 month and 2.3-fold at 2 months, although both differences were at the borderline of signifi-



Plasma levels of human and mouse AGT, TRC, and PRC among offspring of *hREN*×*hAGT* matings. Units for PRC and TRC are ng Ang I · mL⁻¹ · h⁻¹; for AGT, ng Ang I/mL. Measurements were taken at 4 and 8 weeks of age.

cance. These data suggest that mouse prorenin in the plasma is derived largely from the kidney, whereas human prorenin is largely from extrarenal sources.

Discussion

The present study shows that mice doubly transgenic for 45-kb *hREN* and 14-kb *hAGT* genes remain normotensive because they can suppress secretion of both human and mouse renin and thus maintain a constant PRA. In at least 1 previous model, human renin was secreted from extrarenal sites,⁷ and plasma levels of human renin were inappropriately high for the blood pressure. In 45-kb *hREN* mice, human renin is derived exclusively from the kidneys and is secreted normally in parallel with the endogenous mouse renin.⁹ It is this normality of human renin secretion in 45-kb *hREN* mice that enables them to remain normotensive, even with plasma levels of human AGT chronically elevated some 5-fold.

Although plasma levels are determined primarily by secretory mechanisms controlling the release of stored renin, mechanisms regulating renin gene expression may also contribute to the determination of the amount of renin and prorenin release. In this respect, the longer transgene may contain additional regulatory sequences that may affect basal levels, responses to physiological stimuli, or both.

Human plasma renin levels were similar in 45-kb *hREN* mice and in *hREN* lines used in the 2 previous mouse models^{2,3,7,9} (30 to 50 ng Ang I · mL⁻¹ · h⁻¹). However, in 45-kb *hREN* mice doubly transgenic for *hAGT*, PRA was unchanged and hPRC was suppressed up to 3.2-fold. In contrast, in the other 2 lines, PRA was highly elevated in the doubly transgenic mice. Although in 1 of these lines human PRC was suppressed about 2.3-fold, PRA remained high,² suggesting that suppression of human PRC was inadequate. The latter observation is consistent with findings that human renin is expressed in renal juxtaglomerular cells of all transgenic lines. Although this component of circulating renin may be normally regulated, plasma human renin secreted from extrarenal sites is unlikely to respond to increases in blood pressure or Ang II and remains elevated.

It is noteworthy that we used the same *hAGT* line as 1 of the previous studies.³ Although plasma levels of human renin and human AGT were similar in the singly transgenic lines, only the 45-kb *hREN* mice were able to suppress human renin and remain normotensive. Thus, the reason that mice made doubly transgenic for the 13-kb *hREN* transgene became hypertensive was not overexpression of the human renin-angiotensin system per se but rather their inability to suppress renin secretion when their blood pressure rose as a result of the excess angiotensin that was generated.

It is unlikely that the level of AGT plays a significant role in the present model. Although in normal human plasma AGT circulates at levels close to the K_m for its reaction with renin, it is the level of renin that changes rapidly in response to physiological perturbations affecting blood pressure and electrolyte balance. Any increase in AGT should feed back to the kidney to suppress renin secretion.

Human AGT levels in the 14-kb *hAGT* line were about 5-fold higher than normal levels in human plasma. This is similar to the elevated substrate levels observed in many

women taking oral contraceptives.¹³ Despite the elevated substrate, these women remain normotensive, presumably because their plasma renin levels are suppressed through the normal feedback mechanism. Therefore, it is surprising that the M235T mutation in the human AGT gene¹⁴ that appears to be associated with a mere 15% increase in plasma AGT levels should confer an increased risk for hypertension, unless there were some concomitant defect in the mechanisms that regulate renin secretion in these individuals. In this respect, 1 study of the M235T polymorphism that measured PRC rather than PRA levels reported a suppression of PRC in subjects with elevated AGT but found no association of plasma AGT levels with blood pressure.¹⁵

Another study that has implicated a role for AGT in hypertension is the demonstration that titration of plasma AGT levels in mice by gene targeting causes proportional changes in blood pressure.^{16,17} Although plasma renin levels in mice are exceptionally high, mouse AGT circulates at a level ≈40-fold lower than in most other species, including humans, with the result that PRA in mice is the same as in other species. However, with limiting concentrations of AGT, the rate of angiotensin formation in mice is more sensitive to changes in substrate concentration than other species, and mice may not be able to regulate their enormous plasma renin levels sufficiently to compensate for changes in substrate concentration. Thus, the substrate dependence of blood pressure observed in AGT knockout mice may be a peculiarity of the mouse renin-angiotensin system.

In addition to the actual level of AGT, the present study suggests that the tissue source of AGT may not be as important as that of renin. In each of the *hAGT* transgenic lines used to prepare the doubly transgenic animals, *hAGT* mRNA was detected in a number of unusual tissues, such as the heart,^{1,10,18} lung, and ovary^{10,18} and skeletal muscle,¹ sites where AGT is not normally expressed. Nonetheless, if human renin is appropriately regulated, these mice remain normotensive.

In summary, transgenic mice containing an *hREN* gene fragment with long flanking sequences express human renin in a physiologically appropriate manner. When these mice are mated with mice carrying the human AGT gene, the doubly transgenic offspring that express both human renin and human AGT are normotensive, despite high levels of human renin and human AGT in the singly transgenic parental lines. This results because both human and mouse renins are suppressed to maintain a normal PRA.

The significance of this finding is best understood in relation to the role of the kidneys in maintaining blood pressure. The kidneys maintain a constant blood pressure by excreting or retaining sodium as needed. Sodium excretion in turn is regulated by the renin-angiotensin-aldosterone system. Therefore, as long as renin is synthesized and secreted appropriately by the kidneys and adequate amounts of salt are available, normal renal function determines blood pressure, and the activity of the renin-angiotensin system is suppressed. Only when renin secretion escapes normal renal controls will the renin-angiotensin system interfere with normal renal regulation of blood pressure.

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