Increased blood pressure in transgenic mice expressing both human renin and angiotensinogen in the renal proximal tubule

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Lavoie, Julie L., Kristy D. Lake-Bruse, and Curt D. Sigmund. Increased blood pressure in transgenic mice expressing both human renin and angiotensinogen in the renal proximal tubule. Am J Physiol Renal Physiol 286: F965–F971, 2004. First published January 13, 2004; 10.1152/ajprenal.00402.2003.—The purpose of this study was to evaluate the physiological significance of a tissue renin-angiotensin system in the proximal tubule of the kidney. To accomplish this, we produced mice that express human renin (hREN) under the control of the kidney androgen-regulated promoter (KAP), which is androgen responsive. One of the lines expressed the hREN transgene primarily in the kidney. Renal expression of the transgene was undetectable in females but could be induced by testosterone treatment. Because the renin-angiotensin system is species specific, we bred KAP2-hREN mice with the mice expressing human angiotensinogen under the same promoter (KAP-hAGT) to produce offspring that expressed both transgenes. We measured mean arterial blood pressure (MAP) in the carotid artery of double-transgenic and control mice using radiotelemetry. Double-transgenic female mice had a normal baseline MAP (116 ± 4 mmHg, n = 8), which increased by 15 mmHg after 2 wk of testosterone treatment, and returned to baseline after elimination of the testosterone pellet. The change in arterial pressure paralleled the change in plasma testosterone. There was no MAP change in testosterone-treated control littermates. We conclude that dual production of renin and angiotensinogen in the renal proximal tubule can result in a systemic increase in arterial pressure. These data support a role for a tissue-specific renin-angiotensin system in the renal proximal tubule that contributes to the regulation of systemic blood pressure.

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To produce pKAP, the KAP-hAGT construct was modified to allow for a NorI insertion site downstream of the KAP promoter sequence while simultaneously deleting the entire coding potential of hAGT contained within exon II. We first PCR amplified hAGT and inserted a NorI site 42 bp from the end of hAGT exon II, resulting in the deletion of the first 817 bp of exon II including the translational start site (5′ and 3′ primers: 5′-AGCCGCCCTTGGCAAAGGT-GGGT-GAAAGGT GGTTTCCTGTAATGCCT-3′ and 5′-AATGTAATGTTCAGTGTTAGT-GCTTCAATG-3′, respectively). The PCR product was then inserted into pcR2.1, digested with Spel and NorI, and subcloned into pGEM5. We subsequently PCR amplified the KAP promoter and added a NorI cut site 57 bp downstream of BglII (5′ and 3′ primers: 5′-ACATAT-GGAACATAGCGCATCTCCGGCT-3′ and 5′-AGCGGCCGC-TGGACAGCCTCGGTCTTAA-3′, respectively). The PCR product was inserted into pcR2.1 vector (Invitrogen), digested with NorI and NdeI, and subcloned into the pcGEM5 vector containing the modified hAGT. All cloning junctions were confirmed by sequencing.

The final 14.3-kb pKAP2-hREN transgene was excised by digestion with NdeI and Spel, purified by agarose gel electrophoresis, and recovered by gel extraction. The isolated pKAP2-hREN construct was modified by inserting the hREN cDNA into a construct containing the KAP promoter and noncoding DNA encompassing exons 3–5 of hAGT. This was necessary because the hAGT cDNA included exon 5 of hAGT. This was necessary because

Plasma renin assay. Plasma human renin concentration (PRC) and plasma mouse renin activity (PRA) were determined in male mice as described previously (40). Radioimmunoassay was performed on plasma using an ANG 1-129-labeled RIA kit (PerkinElmer Life Sciences). Samples were diluted with reagent blank to remain on the linear portion of the standard curve.

Immunohistochemistry. Male mice were euthanized by CO2 asphyxiation and then perfused transcardially with 20 ml of PBS followed by 50 ml of 4% paraformaldehyde in PBS. The kidneys were removed, postfixed at 4°C for 3–4 h, and then placed in 30% sucrose solution at 4°C. The following day the kidney was frozen and cut longitudinally (10 μm) using a Microm cryostat. The slices were incubated at 4°C for 18 h with rabbit polyclonal antisera to hRR (generous gift from Walter Fischli, Hoffmann-LaRoche, Basel, Switzerland). The sections were then incubated with anti-rabbit biotinylated antibody (1:10,000; Jackson ImmunoResearch Lab) at room temperature for 2 h. The section was then incubated with rhodamine-streptavidin IgG (1:100 dilution; Jackson ImmunoResearch Lab) at room temperature for 1 h. Slides were visualized using a Nikon eclipse E600 fluorescence microscope equipped with a SPOT RT digital camera (Diagnostic Instruments). All photographs were taken at the same magnification and exposure.

Physiology. Mice were anesthetized with pentobarbital sodium (50 mg/kg ip) and surgically implanted with TAI11A-CA20 radiotelemeters (Data Sciences International, St. Paul, MN) in the left carotid artery for direct measurement of arterial pressure (AP) and heart rate (HR), as described previously (39). After surgery, an analgesic, bupivacaine HCl (0.25%, Abbott Laboratories, Chicago, IL), was applied topically to the incision. Mice were given 7 days to recover, after which time AP and HR were continuously recorded for 3 days. Females were then subcutaneously implanted with a 5-mg testosterone pellet (A-151, Innovative Research of America, Sarasota, FL), as described previously (10), and AP and HR were recorded for an additional 22 days until blood pressure returned to baseline. The testosterone pellets used were designed for 21-day release. All data were collected and stored using Dataquest ART. MAP and HR were determined by averaging all acquired data into 12-h blocks paralleling the light-dark cycle.

Plasma testosterone assay. Blood samples were taken from male and female mice before and at 15 and 25 days after administration of a 5-mg testosterone pellet. Using microhematocrit tubes (VWR Scientific), samples were collected from the suborbital sinus into tubes containing 0.5 M EDTA, centrifuged at 4,000 rpm for 10 min at 4°C, and then stored at −80°C. Plasma testosterone levels were determined using a radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX). This RIA kit has been previously validated for the measurement of circulating testosterone in mice (19).

Statistical analysis. Data are expressed as means ± SE. Group comparisons were made using one-way ANOVA and a Tukey post hoc test. A value of P < 0.05 was considered statistically significant.

RESULTS

KAP2-hREN transgenic mice were generated to produce a specific and androgen-inducible expression of hREN in the proximal tubules of the kidney. This was accomplished by inserting the hREN cDNA into a construct containing the KAP promoter and noncoding DNA encompassing exons 3–5 of hAGT (Fig. 1). In our initial experiments, we simply fused the KAP promoter to hREN cDNA but did not achieve any expression of the transgene in these mice. To correct this problem, we designed a new construct termed pKAP2, which includes exons 3–5 of hAGT. This was necessary because evidence suggests that this KAP promoter may require enhancer elements that are present downstream of the hAGT (1). Exons 3–5 of hAGT, in the context of this construct, are not
encoded into protein and only supply the distal enhancer and exon-intron splice sites. We generated four transgenic founders.

Transgene expression was evaluated in kidney, heart, liver, lung, spleen, submandibular gland, adrenal glands, testes, epididymis, brain, white/brown fat, and skeletal muscle using RNase protection. Three of the four lines were found to express the transgene mRNA (data not shown). Expression was detected in the kidney of all three lines but was variable in other tissues. Tissue-specific expression was the most restricted in line 12403/1, where we found it mainly in the kidney and brain (Fig. 2A). Although expression of hREN was higher in the other lines, we felt it important to sacrifice the level of expression for greater kidney specificity. To examine the inducibility of the KAP promoter, transgenic female mice from this same line were implanted with a testosterone pellet (0.24 mg/day). After 7 days the mice were euthanized, and tissues were harvested for further analysis of hREN gene expression. Baseline expression of the hREN construct was undetectable in any of the tissues examined (Fig. 2B). Testosterone strongly induced expression of the hREN gene in the kidney and, surprisingly, in the brain (Fig. 2B). Much lower level induction was evident in the liver, aorta, and brown adipose tissue.

Immunohistochemistry was performed using an hREN antibody and kidney sections from nontransgenic mice as controls (Fig. 3). Although there was a generalized background in both transgenic (Fig. 3, A and B) and control (Fig. 3C) sections, the transgenic sections showed increased focal staining for hREN in renal tubules. This is consistent with the low level of hREN expression detected by RNase protection. In addition, occasional strong staining for hREN was observed in epithelial cells in the tubular wall from sections from transgenic mice (arrowheads in Fig. 3). Expression of the transgene was evident in all segments of the proximal tubule. No expression was observed in blood vessels, glomeruli, or juxtaglomerular cells.

Given the presence of ectopic expression of hREN in extra-renal tissues, we were concerned that hREN might be released in the systemic circulation. Therefore, we measured plasma hREN concentration in single KAP2-hREN transgenic mice in the presence of excess exogenous hAGT to determine whether extrarenal transgene expression resulted in a significant increase in hREN in the systemic circulation. There was no significant increase in mouse plasma renin activity in transgenic compared with nontransgenic mice (Fig. 4). We also found no significant increase in human renin in the plasma of KAP2-hREN mice over the background levels observed in nontransgenic controls. Accordingly, it is unlikely that a significant amount of hREN protein is released into the systemic circulation in this transgenic model.

To determine the physiological significance of an increased RAS in the kidney proximal tubule regarding blood pressure, we generated KAP2-hREN/KAP-hAGT double-transgenic mice. This was necessary given the strict species specificity of

Fig. 2. Transgene expression. RNase protection assay showing tissue expression of hREN RNA in a male KAP2-hREN transgenic mouse (A) and testosterone-inducible expression of hREN in a female transgenic mouse (B). The hREN and β-actin fragments are indicated. Female transgenic mice treated and untreated with testosterone are indicated by a T or −, respectively. A nontransgenic control from the kidney is labeled as −/− (negative transgenic and negative testosterone). K, kidney; Wa, white adipose tissue; Br, brain; T, testis; Ba, brown adipose tissue; Sm, skeletal muscle; Sg, submandibular gland; D, diaphragm; Lv, liver; Hr, heart; Ov, ovary; Ut, uterus; Lg, lung; Ao, aorta.

Fig. 3. Cell-specific expression of the KAP2-hREN transgene. Epifluorescence images (×40 magnification) of kidney from KAP2-hREN/KAP-hAGT (A and B) and nontransgenic (C) mice are shown. Arrowheads indicate strong hREN staining in epithelial cells of the tubular wall.

Fig. 4. Plasma renin activity (PRA) and concentration (PRC). Shown is mouse PRA (filled bars) and human PRC (gray bars) as determined by a RIA of plasma from KAP2-hREN male transgenic mice (n = 8) compared with their nontransgenic littermates (n = 5). Values are means ± SE.
the renin-AGT biochemical reaction (12). We found no differences in daytime or nighttime MAP and HR when comparing male [daytime: 113 ± 2 mmHg, 533 ± 6 beats/min (bpm); nighttime: 123 ± 1 mmHg, 578 ± 10 bpm] or female (daytime: 100 ± 3 mmHg, 572 ± 13 bpm; nighttime: 114 ± 1 mmHg, 623 ± 12 bpm) double-transgenic mice with their nontransgenic male (daytime: 114 ± 3 mmHg, 538 ± 26 bpm; nighttime: 123 ± 1 mmHg, 578 ± 25 bpm) or female (daytime: 103 ± 1 mmHg, 582 ± 15 bpm; nighttime: 117 ± 3 mmHg, 640 ± 9 bpm) littermates (Fig. 5). In addition, there was no significant difference in the rise in blood pressure recorded during the day or at night. The increase in blood pressure in the double-transgenic females was not observed in their nontransgenic littermates (○) concurrently treated with testosterone and disappeared once the testosterone pellet was eliminated in the double-transgenic females (Fig. 7). The changes in MAP paralleled the changes in plasma testosterone concentration (Fig. 8). Testosterone levels in the female testosterone-treated double-transgenic mice were similar to the levels found in male mice at baseline.

Because the male double-transgenic mice had no blood pressure phenotype, we examined kidney expression of both human and mouse REN in comparing male and female double-transgenic and nontransgenic mice (Fig. 9). Human REN expression was similar in male vs. testosterone-treated female double-transgenic mice. On the contrary, mouse REN was markedly blunted in the KAP2-hREN/KAP-hAGT male mice compared with their nontransgenic littermates (Fig. 9). Interestingly, there was no blunting of mouse renin expression in KAP2-hREN/KAP-hAGT female mice treated with testosterone, likely accounting for the gender-specific increase in arterial pressure.

**DISCUSSION**

The renal proximal tubule is thought to contain all the components of the RAS. Intrarenal AGT seems to be mainly localized in the proximal tubule (7, 11, 14, 31), and ACE has been found to be associated with the brush borders of the proximal tubule (33, 35), whereas low-level concentrations of renin have been detected in the proximal tubule fluid (18). Thus locally produced ANG II could stimulate AT1 receptors present on the brush borders and basolateral membrane of the proximal tubules. It has been suggested that the stimulation of AT1 receptors in the proximal tubule has direct effects on sodium transport in the early nephron by stimulating sodium-hydrogen exchange in the proximal tubule and indirect effects in the late nephron by regulating synthesis of epithelial sodium channels by aldosterone (4, 32).

To examine the relative importance of the RAS in the proximal tubule, we previously generated transgenic mice expressing hAGT in the renal proximal tubule (9). This was accomplished by driving expression of hAGT with a proximal tubule-specific and androgen-regulated promoter (22). Expression of the transgene was restricted to the kidney and, within the kidney, expression was restricted to proximal tubule cells. Biochemical and physiological studies confirmed that there was no secretion of hAGT into the systemic circulation. In females, expression of hAGT could be induced by androgen. Because of the species-specific interaction between renin and angiotensinogen, we initially bred these mice with transgenic mice expressing the human renin gene systemically. Within the kidney, hREN was expressed in juxtaglomerular (JG) cells with little evidence for expression in the proximal tubule. The MAP in single and nontransgenic mice was normal. However, the double-transgenic mice were moderately hypertensive, demonstrating that the increase in pressure was dependent on the production of ANG II through the enzymatic interaction of hREN and hAGT (8). Moreover, the blood pressure of the double-transgenic mice could be lowered by losartan, although at a higher dose than required to lower blood pressure in mice with elevated circulating ANG II. Interestingly, although the increase in blood pressure was dependent on ANG II, there was no elevation in plasma ANG II, suggesting an intrarenal mechanism. This was the first demonstration of systemic hypertension from a purely renal effect of locally generated ANG II.

Although the actual mechanism by which blood pressure increases in the model remains unclear, the current study prompted us to ask how ANG II is generated in the kidney of the double-transgenic mice? We presumed that because hAGT was secreted into the lumen of the proximal tubule (20), the site of ANG II production must be in the tubular lumen of the
proximal tubule or further distal along the nephron. Given the primary expression of renin in the JG cells, this led to the question of the source of renin needed to cleave intratubular AGT. We considered three potential sources. The first possibility is that hREN enters the tubular lumen by filtration. Its molecular mass of ~42,000 Da is at the upper end of the range for a filtered product. The second possibility was facilitated transport from the renal interstitium. Renin is released from the JG cells into the interstitial space in the kidney and thus is in close proximity to the basolateral membrane of the proximal tubule. Although no specific transport mechanism has been proposed in the proximal tubule, there is evidence for renin uptake in other tissues (6), and renin binding proteins and a renin receptor have been recently described (2, 28). Finally, the notion of direct synthesis of renin by the proximal tubule must be considered. Indeed, Moe et al. (24) reported renin synthesis in the proximal tubule. Although we have not found evidence for proximal tubular production of hREN in the transgenic mice used in those studies, we cannot rule out the possibility that it is made and secreted in small amounts.

To address the issue of proximal tubule production of renin and to answer the question of whether there is a potential for intratubular ANG II generation from REN and AGT produced in the proximal tubule, we developed a novel transgenic mouse model that expresses hREN from the KAP promoter. This is the same androgen-dependent promoter used to express AGT. An RNase protection assay revealed hREN to be mainly present in the kidney in the male KAP2-hREN mice, whereas in females, expression in kidney and brain could only be found after treatment with testosterone. In the kidney, we used immunohistochemistry to confirm that hREN expression was specifically targeted to the proximal tubule. Given the presence of ectopic expression of hREN, we confirmed its absence in the circulation biochemically. We then bred the KAP2-hREN mice with the KAP-hAGT mice described above. The use of androgen-responsive transgenes provided an opportunity to examine the effects of both acute and chronic overexpression of the RAS in the proximal tubule.

Females treated with testosterone exhibited a 15-mmHg increase in MAP, which reached a plateau at 15 days. The peak of the blood pressure response and its return to baseline correlated with the increase and then return to baseline of plasma testosterone. These data strongly argue that intratubular generation of ANG II from REN and AGT secreted from the proximal tubule can have a significant effect on systemic blood pressure. It remains unclear whether ANG I is first generated within proximal tubule cells from intracellular cleavage of hAGT by hREN. An intracellular pathway for ANG II biosynthesis has been proposed, and evidence for an intracellular form of renin has been observed in adrenal gland and brain, although no evidence has been found in the kidney (5, 17, 38). If ANG II is indeed generated in the tubular fluid, it remains unclear whether it occurs primarily in the proximal tubule or in more distal segments. Another remaining issue is whether the increase in blood pressure is due to ANG II-mediated stimulation of sodium transport in the proximal tubule, perhaps through activation of the sodium/hydrogen exchanger, or by

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**Fig. 7.** MAP in testosterone-treated female double-transgenic mice. Shown is the compiled MAP in female double-transgenic mice (filled bars, n = 8) compared with their nontransgenic littermates (gray bars, n = 7) during the day (left) and night (right) before and at days 14–18 and 25 after the administration of a testosterone pellet. Values are means ± SE expressed as an averaged 12-h block. *P < 0.05 compared with all other conditions and nontransgenic littermates.

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**Fig. 8.** Plasma testosterone. Shown is the plasma testosterone level in male (M; gray bar, n = 4) and in female (filled bars, n = 5) KAP2-hREN transgenic mice. The levels shown in female are before and at 15 and 25 days after the administration of a testosterone pellet to female double-transgenic mice.

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**Fig. 9.** hREN and mouse renin (mREN) expression. RNase protection assay is shown of kidney expression of mREN (left) and hREN (right) RNA in a male and testosterone-treated female double-transgenic (+) mouse compared with nontransgenic littermates (−). M, male; F+T, female treated with testosterone.
activation of epithelial sodium channels in collecting duct cells. Overexpression of the sodium/hydrogen exchanger in renal tubules results in salt-sensitive hypertension (16).

Surprisingly, male KAP2-hREN/KAP-hAGT mice had no blood pressure phenotype. Because the levels of testosterone and kidney hREN are comparable in male and testosterone-treated female double-transgenic mice, we attribute this lack of blood pressure phenotype in the males to a decrease in endogenous mouse renin. It is unclear why there was no similar downregulation of endogenous mouse renin mRNA in female double-transgenic mice treated with testosterone. This difference might be due, in part, to the acute affects of the testosterone treatment. Indeed, a maximal increase in blood pressure is obtained after 12–14 days of testosterone treatment and then maintained for only 3–4 days, which may not be sufficient to cause a significant downregulation of the endogenous renin gene. In addition, there may be developmental effects that might be in play because the male double-transgenic mice have an inherent increase in proximal tubule ANG II due to presence of hREN and hAGT. Nevertheless, that the testosterone-treated females had elevated blood pressure and a retention of endogenous mouse renin expression (and presumably circulating mouse renin) suggests that the mechanism of the blood pressure elevation may be more complicated than explained by a renal mechanism alone. Indeed, it will be important to experimentally distinguish between the blood pressure effects caused by either J) the combined actions of mouse and human ANG II in the kidney (a dose-dependent effect of ANG II) or 2) the combined effects of intrarenal human ANG II and circulating mouse ANG II (suggesting both renal and peripheral mechanisms).

It is also important to point out that although hREN expression was evident in the brain of testosterone-treated females, the absence of hAGT in the brain and plasma (9) likely eliminates a central mechanism for the blood pressure elevation observed in the model. We have previously demonstrated that expression of both hREN and hAGT in the brain is absolutely required to cause a centrally mediated rise in arterial pressure (25–27).

Finally, it is worth mentioning that the design of the KAP2-hREN transgene construct, which is inserting hREN in place of the coding region of hAGT while retaining downstream (non-coding) exons, was the product of studies suggesting that a transcriptional enhancer in exon 5 and the 3′-untranslated region of the hAGT gene cooperated with the KAP promoter to drive proximal tubule and androgen-dependent expression (1). Constructs containing only the KAP promoter segment fused to reporter genes such as β-Gal and luciferase failed to drive proximal tubule-specific expression (Sigmund CD, unpublished observations).

In conclusion, our data strongly suggest that proximal tubule RAS can have a significant effect on blood pressure. This effect may be caused by alterations in sodium or fluid homeostasis, perhaps through alterations in transport mechanisms in the kidney. Of clinical relevance, such effects appear to be a common underlying mechanism causing high blood pressure in a number of human genetic syndromes (34, 37). We are presently in the process of studying mice that overexpress the type 3 sodium/hydrogen exchanger driven by the same KAP2 promoter to elucidate the role of sodium-hydrogen exchange in the proximal tubule in blood pressure regulation.

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