Lower Blood Pressure in Floxed Angiotensinogen Mice After Adenoviral Delivery of Cre-Recombinase

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Abstract—Recent experimental evidence suggests a role for tissue renin-angiotensin systems in the development of hypertension. To test the importance of tissue renin-angiotensin systems in the development and maintenance of angiotensin II-dependent hypertension, we generated a transgenic model in which exon 2 of the human angiotensinogen gene is flanked by loxP sites (hAGT\(^{\text{flox}}\)) so that this region of the gene can be deleted by the cre-recombinase. Double transgenic human renin and hAGT\(^{\text{flox}}\) (R\(^{+/}\)/A\(^{+/}\)) mice of two independent lines exhibited elevated blood pressure. Acute administration of an adenovirus containing cre-recombinase (Adcre) lowered blood pressure by 30 mm Hg over a 4-day period as measured with fluid filled catheters. The chronic effect of Adcre administration on blood pressure was determined by radiotelemetry in a separate group of R\(^{+/}\)/A\(^{+/}\) flox mice. Blood pressure decreased by 25 mm Hg from baseline by day 8 post-Adcre, but increased on each day thereafter until it was 90% of baseline by day 21 post-Adcre. Expression analysis indicated the absence of detectable hAGT mRNA in the liver at day 5 post-Adcre, but reappeared at normal levels at days 14 to 21 post-Adcre. These studies suggest that Adcre is effective for acute, but not chronic, elimination of hepatic hAGT. Chronic elimination of hepatic hAGT will likely require the use of transgenic mice endogenously expressing cre-recombinase in the liver. (Hypertension. 2002;39[part 2]:629-633.)

Key Words: gene expression • mice • renin-angiotensin system

Several models of angiotensin II (Ang II)–dependent hypertension have been described in transgenic rats and mice.\(^1\)\(^-\)\(^3\) Previously, we developed a model of Ang II–dependent hypertension by expressing both the human renin (hREN) and angiotensinogen genes (hAGT) in mice.\(^3\) hREN/hAGT double transgenic mice (R\(^{+/}\)/A\(^{+/}\)) exhibit increased blood pressure and elevated levels of circulating Ang II. However, recent studies by our laboratory suggest that alterations in tissue renin-angiotensin systems (RAS) may be involved in the development and maintenance of hypertension in this model. Tissue RAS are hypothesized to exist in tissues (such as the heart, kidney, brain, and vasculature) capable of generating and responding to Ang II and have been implicated in the pathogenesis of hypertension, because inhibitors of the RAS are effective antihypertensive agents even in patients with normal or low plasma renin activity, an index of circulating RAS function.\(^4\)\(^-\)\(^6\) In hAGT mice, AGT is expressed in liver (the source of circulating AGT) and in kidney, heart, brain, and adipose tissue, thus providing a source of substrate in many tissues.\(^7\) Evidence supporting a role for tissue RAS in the model include a greater blood pressure sensitivity to intracerebroventricular infusion of losartan than normotensive mice, thus suggesting that Ang II generation and action in the brain may play an important role in blood pressure regulation in this model.\(^8\) Moreover, in a separate series of studies, we reported that proximal tubule-specific or brain-specific overexpression of the hAGT gene is able to increase blood pressure in the absence of an increase in plasma Ang II.\(^9\)\(^-\)\(^11\) Since the level of AGT expression is high in the kidney of hAGT transgenic mice, a kidney-specific mechanism may also contribute to the mechanisms of hypertension in R\(^{+/}\)/A\(^{+/}\) mice.\(^7\)

To test the role of tissue RAS in the development and maintenance of hypertension in the R\(^{+/}\)/A\(^{+/}\) model, we created a transgenic mouse in which exon-2 of the hAGT was flanked by loxP sites (hAGT\(^{\text{flox}}\)). We previously demonstrated liver-specific deletion of the HAGT\(^{\text{flox}}\) transgene in mice given an intravenous injection of a cre-recombinase containing adenovirus (Adcre).\(^12\) The deletion of the hAGT\(^{\text{flox}}\) transgene in the liver was associated with a significant decrease in the plasma level of hAGT (>90% of control levels) and a markedly depressed or absent pressor response to infusion of purified recombinant human renin protein. These results demonstrated that deletion of the liver hAGT by intravenous administration of Adcre can acutely eliminate hAGT protein from the plasma of hAGT\(^{\text{flox}}\) mice. The goal of this study was to determine whether double transgenic R\(^{+/}\)/A\(^{+/}\) flox mice exhibit a hypertensive phenotype and to determine the effect of chronic administration of Adcre on blood pressure using continuous monitoring of arterial pressure by radiotelemetry.
Methods

Animals
Studies were performed on 16- to 20-week-old mice fed standard mouse chow and water ad libitum. Care of the mice met or exceeded the standards set forth by the National Institutes of Health in Guidelines for Care and Use of Experimental Animals. All procedures were approved by the University Animal Care and Use Committee.

Implantation of Fluid-Filled Catheters and Recording of Blood Pressure
Mice were anesthetized with sodium pentobarbital (50 mg/kg) and catheters placed into the left common carotid artery and right jugular vein. Mice were allowed 48 hours to recover before blood pressure was measured. Catheters were flushed daily with sterile heparinized saline (500 U/mL). Blood pressure was measured for 2 hours per day in conscious, freely-moving mice using a PowerLab 4/sp polygraph (AD Instruments) and acquired on a computer running Chart 4 software. Adcre (driven by the cytomegalovirus [CMV] promoter) was administered via the venous catheter after the second control day (1.1 x 10^11 plaque-forming units in 0.1 mL) and blood pressure was then measured each day for the next 4 days. Construction of Adcre was previously described.13 The acute effect of Adcre administration on blood pressure was determined in a separate group of R/\( ^{+/+} \) mice (line R/\( ^{+/+} \)/H11001).

Implantation of Telemetry Probes and Recording of Blood Pressure by Telemetry
Mice were anesthetized with sodium pentobarbital (50 mg/kg), the catheter (Data Sciences International) was placed into the left common carotid artery,13 and the transmitter was placed subcutaneously along the left flank.14 Mice were given 48 hours to recover, after which time heart rate and arterial pressure were continuously recorded (sampling every 5-minute for 20-second intervals). Data were collected and stored using Dataquest ART. Basal blood pressure was collected for 3 days, after which they were anesthetized with sodium pentobarbital (50 mg/kg) and a catheter (PE10) was inserted into the right jugular vein for administration of Adcre. The catheter was removed and the vein tied off with two silk ligatures (6-0). Mice were allowed to recover until blood pressure reached baseline (6-8 hours), and then were continuously monitored for 21 days. Blood pressure was determined by the average of each individual sampling segment over a 24-hour period.

RNase Protection Assay
A commercially available RNase protection assay kit (RPA III, Ambion) was used to quantify hAGT mRNA in 15 \( \mu \)g of total tissue RNA. Probes for hAGT exon-2 or -5 were partial cDNA sequences amplified by reverse transcriptase polymerase chain reaction and cloned into pCR2.1 (Invitrogen). For liver, a 204-bp fragment (coordinates 339 to 542) was used to detect exon-2, and a 236-bp fragment (coordinates 1531 to 1766) was used to detect exon-5. For kidney, a 352-bp fragment (coordinates 191 to 542) was used to detect exon-2. The 28S rRNA (protected fragment 115 bp) was used as the housekeeping control in all assays (template obtained from Ambion).

Statistical Analysis
All blood pressure data are presented as mean±SE. Statistical analysis was performed by Student’s \( t \) test or by ANOVA using the SigmaStat software package.

Results
Blood pressure was measured in two lines of hAGT\( ^{+/+} \) mice that exhibited marked differences in the plasma level of hAGT and the magnitude of expression of hAGT mRNA in the liver.12 The 4284/1 line exhibits a 20-fold higher plasma level of hAGT protein than the 4258/1 line. Both lines were bred with mice expressing the same hREN transgene.13 Despite the difference in hAGT expression, the blood pressure of double transgenic mice derived from either line was similar and averaged 157±3 mm Hg versus 122±2 mm Hg (4284/1), and 155±3.3 mm Hg versus 116±3 mm Hg (4258/1), double transgenic versus control, respectively (Figure 1).

We next determined the effect of Adcre on blood pressure in double transgenic mice from the 4258/1 line. Baseline blood pressure averaged 161±7 mm Hg for the control period and was not significantly changed on days 1 (159±8 mm Hg) and 2 (160±6 mm Hg) after administration of Adcre (Figure 2). The blood pressure significantly fell by 29 and 33 mm Hg on days 3 (131±8 mm Hg) and 4 (127±8 mm Hg) post-Adcre.

To extend our finding that Adcre can acutely decrease blood pressure in this model, we needed to develop a methodology that would allow for the long-term measurement of blood pressure. We therefore used radiotelemetry to record blood pressure for a period up to 21 days post-Adcre in a separate group of R/\( ^{+/+} \) mice from the 4258/1 line. Procedures for the implantation and validation of radiotelemetry in mice have been reported.13,14 In particular, we were interested in determining whether blood pressure would remain low chronically or gradually rise after administration of Adcre. Twenty-four hour blood pressure in six individual mice is shown in Figure 3A. Baseline blood pressure ranged from 148 to 166 mm Hg. A significant drop in blood pressure was observed in 5 mice after Adcre, whereas 1 mouse (No. 12947/1) exhibited little decrease in blood pressure in response to Adcre. In 2 mice (Nos. 12716/3 and 14782/2), shorter blood pressure measurements were obtained owing to

Figure 1. Baseline arterial pressure in R/\( ^{+/+} \) mice. Resting MAP in R/\( ^{+/+} \) mice in line 4258/1 (filled bar, n=7) and line 4284/1 (filled bar, n=3) and their littermate controls (open bars, n=8 for 4258/1 and n=4 for 4284/1) was measured by fluid-filled catheter. * \( P<0.05 \) vs controls.

Figure 2. Acute arterial pressure response to Adcre. MAP was measured by fluid-filled catheter before and each day after intravenous administration of Adcre (n=4). The time of Adcre administration is indicated. * \( P<0.05 \) vs pretreatment baseline arterial pressure.
telemeter failure (battery failure in one and transmitter failure in the other). The maximum depressor response to Adcre administration occurred between days 4 and 8 post-Adcre, at which time blood pressure gradually began to rise in each mouse. Importantly, administration of an adenovirus contain the bacterial lacZ gene (Adβgal) had no effect on blood pressure in R7/A15 mice over a 14-day period (data not shown).

A summary of the group data are shown in Figure 3B. Twenty-four-hour blood pressure for the whole group over the 3-day control period averaged 163±3 mm Hg and decreased to 145±6 mm Hg on day 2 post-Adcre. Blood pressure decreased to 138±4 mm Hg on day 8 post-Adcre, after which time it began to increase daily until it averaged 151±2 mm Hg on day 21 post-Adcre.

We also examined the 24-hour circadian rhythms of mean arterial pressure under baseline conditions and after administration of Adcre (Figure 4). A modest circadian rhythm was observed under control conditions with daytime and nighttime mean arterial pressures averaging 160±5 and 170±4 mm Hg. The magnitude of the circadian rhythm appears enhanced after administration of Adcre. Likely, this occurred because the control data were obtained 2 days after surgery whereas normal circadian rhythm can take up to 7 days post surgery to return to normal.14 Despite this, administration of Adcre clearly decreased both daytime (129±4 mm Hg) and nighttime (152±4) mean arterial pressure on day 9 post-Adcre. Although both parameters gradually increased over the next two weeks, only the nocturnal blood pressure nearly reached the pretreatment baseline (163±5 mm Hg), whereas a modest decrease in daytime blood pressure was retained (139±4 versus 160±5 mm Hg).

We considered that the increase in blood pressure after the initial Adcre-mediated drop may be due to several mechanisms. First, it could reflect the activation of tissue RAS pathways. Indeed, even after depletion of hepatic and circulating AGT, AGT would remain in kidney and brain (among other tissues). AGT over-expression in either tissue alone can cause a rise in blood pressure.9,11 Second, the blood pressure elevation may reflect restoration of the circulating RAS, due either to loss of cre expression long term, or repopulation of the liver by AGT expressing hepatocytes. Recall that circulating AGT is almost exclusively derived from the liver.12 To distinguish these possibilities, we determined the level of hAGT mRNA in the liver and kidney of control, Adβgal, and Adcre treated mice. The exon-2 probe hybridizes to a region of hAGT mRNA deleted by cre-recombinase, while the exon 5 probe detects a region of hAGT mRNA retained even after deletion. We previously reported that a stable hAGT mRNA is retained (but lacking exon-2) after cre-mediated recombination.12 Although the hAGT mRNA in the liver was ablated after acute (5-day) administration of Adcre, there was no difference in the level of hAGT mRNA in the liver of chronic Adcre and Adβgal treated mice (Figure 5).

As expected, no significant differences in the levels of kidney hAGT mRNA were detected between the groups. These data suggest that the increase in blood pressure is most likely due to repopulation of the liver with hepatocytes containing an intact hAGT gene.

Discussion

RAS is thought to regulate blood pressure through several mechanisms, including its effects in the systemic vasculature, kidney, and brain. Recent studies have indicated that several of these tissues have the capacity to generate Ang II locally, but the relative importance of the systemic versus tissue RAS in the regulation of blood pressure has yet to be fully understood.16,17 We have previously generated a model of Ang II-dependent hypertension by expressing both the hREN and hAGT genes in transgenic mice.13 The double transgenic mice exhibit increased blood pressure and elevated levels of circulating Ang II, which we initially hypothesized was the cause of the increased blood pressure in this model. However, recent experimental evidence in this model suggests that a tissue RAS in the brain may be involved in the development and maintenance of hypertension.8 In addition, R7/A15 mice exhibit severe endothelial dysfunction that may also play a role in the maintenance of hypertension long term.18

Figure 3. Chronic arterial pressure response to Adcre. MAP was measured by radiotelemetry continuously for 3 days before and for up to 21 days after intravenous administration of Adcre (n=6). A 24-hour blood pressure mean is indicated. The time of Adcre administration is indicated as time = 0. A, Blood pressure response in each of 6 individual mice. B, Summary data from the group (n=6).

Figure 4. Circadian rhythm of arterial pressure in response to Adcre. Twelve-hour arterial pressure during the light (white background) and dark (yellow background) cycles was measured before (red line) and after (blue line) Adcre (n=6).
We have used two approaches to experimentally separate tissue RAS from the circulating RAS. First, we used cell-specific promoters to specifically target expression of RAS components to specific cell types. Using this approach, we demonstrated that specific overexpression of AGT in the kidney or brain can cause hypertension without any elevation in plasma AGT or Ang II. The second approach was to generate cell-specific knockouts of RAS components and assess the physiological consequences of their loss on cardiovascular function. To this end, we developed transgenic mice in which we could perform tissue-specific deletion of the hAGT gene using the cre-loxP recombinase system (hAGTflx).12

We determined basal blood pressure in two lines of double transgenic R /A−/flx mice that markedly differed in the level of expression of the hAGTflx transgene and plasma levels of hAGT protein. One line, 4284/1, exhibits high level hAGT expression in the liver and has plasma levels of hAGT protein that are 20 times higher than that of the lower expressing line, 4258/1. Despite this, double transgenic R /A−/flx mice derived from each line exhibited an equivalent rise in baseline blood pressure. It is possible that the level of hREN protein in the plasma is rate limiting in the generation of Ang II from hAGT in vivo, and thus both lines have similar levels of plasma Ang II. Alternatively, the plasma level of Ang II in each line, while different, may be sufficiently above the plateau needed to increase blood pressure.

Since we determined that double transgenic mice containing the hAGTflx transgene were hypertensive, our next goal was to determine the effect of elimination of circulating hAGT protein on blood pressure in this model by specifically deleting the hAGTflx transgene in the liver. We have previously reported that deletion of the hAGTflx transgene in the liver significantly decreases the levels of detectable hAGT protein in the plasma, and that extrahepatic tissues do not make a significant contribution to circulating hAGT levels.13 These studies were performed in the 4258/1 line because of the more physiologically relevant levels of hAGT in the plasma. When measured using fluid-filled catheters, administration of Adcre significantly decreased blood pressure by ∼30 mm Hg on day 3 after administration. This pattern of blood pressure reduction mirrored the effect of Adcre on plasma levels of hAGT protein that was previously observed in single transgenic hAGTflx mice.12

To determine whether the tissue RAS would be able to increase blood pressure in the absence of elevated circulating levels of Ang II, we needed to perform chronic blood pressure measurements in Adcre-treated R /A−/flx mice. This aim was accomplished by the use of radiotelemetry, which allowed us to measure 24-hour blood pressure continuously for 21 days after Adcre administration. Using this method, we were able to detect a decrease in blood pressure on day 2 post-Adcre. This fall is not due to adenovirus administration, as there was no decrease in arterial pressure after administration of Adβgal. Blood pressure continued to fall until day 8 post-Adcre, at which time it increased daily until it was back to 90% of control by day 21 post-Adcre. It is interesting to note that the blood pressure response to Adcre administration was detected earlier in the animals instrumented with telemeters than in those instrumented with fluid-filled catheters. This may be due to the different sampling periods of the 2 methods. The telemetry readings are the average of a 24-hour period, while blood pressure was measured for a single 2-hour interval each morning with the fluid-filled catheters.

The increase in blood pressure after day 8 post-Adcre could potentially be explained by two different mechanisms. The first would be increases in blood pressure due to the effects of tissue RAS and the second mechanism would be turnover of hepatocytes in the liver that would restore the circulating levels of hAGT thus increasing circulating Ang II. We attempted to differentiate between these two alternatives by examining the levels of hAGT mRNA in the liver at the conclusion of the chronic experimental protocol. Absence of detectable levels of hAGT mRNA in the liver would lead us to hypothesize that the observed increase in blood pressure may be due to the actions of the tissue RAS, whereas the presence of hAGT mRNA would suggest restoration of hAGT-expressing hepatocytes and circulating hAGT. Previous data, as well as data in the acute Adcre mice indicate the absence of hAGT mRNA on days 5 to 7 post-Adcre administration. The absence of hAGT mRNA in the liver correlates with the reduction of blood pressure in Adcre-treated mice as measured by both techniques. Further analysis of hAGT mRNA levels in the chronic Adcre-treated mice revealed the presence of normal levels of hepatic hAGT mRNA on days 19 to 21. This would suggest that the increase in blood pressure observed in these mice is likely due to regeneration of the hAGT mRNA in the liver, increase in the plasma levels of hAGT, and the subsequent increase in circulating levels of Ang II.

Given the presence of normal levels of hAGT mRNA in the liver, we believe that the liver became repopulated with hAGT-
expressing hepatocytes. Although we cannot rule out loss of Adcre as a mechanism, previous studies using adenoviral therapy to target the liver have documented gene expression well past 21 days. Moreover, expression of cre-recombinase should only be required transiently, since the genetic modification in the hepatocytes genome should be permanent and thus irreparable. Recent studies have indicated that the high levels of cre-recombinase expression in mammalian cells can lead to DNA damage and growth inhibition due to recombination at pseudoxP sites present in the mammalian genome. Since the expression of the cre-recombinase in the Adcre vector is under the control of the CMV promoter, it is possible that the high level of cre expression leads to increased turnover of infected hepatocytes and replenishment with hepatocytes that contain a normal hAGT transgene. High levels of cre-recombinase are easily detected in Adcre-infected liver cells (D.E. Stec and C.D. Sigmund, unpublished data). A transgenic model using the albumin promoter to endogenously express the cre-recombinase has previously been reported to cause the efficient deletion of a floxed glucokinase gene. Expression of cre-recombinase in this model was only detected by reverse transcriptase polymerase chain reaction generating a radiolabeled polymerase chain reaction product, and there was no indication of altered hepatocyte morphology or function in these transgenic mice. The use of a transgenic mouse expressing cre-recombinase in the liver chronically would be the next logical step in these experiments.

In conclusion, acute deletion of the hAGT 

transgene in the liver of double transgenic R 

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-12lox mice causes a reduction in blood pressure that lasts for 8 days after administration, at which time the blood pressure increases daily until it is nearly increased to control levels at day 21 after administration. This increase in blood pressure is presumably due to regeneration of hAGT expression in the liver and restoration of plasma Ang II levels. It seems that the use of adenoviral gene therapy to delete the hAGT 

transgene in the liver is not a viable method to determine the chronic effect of the tissue RAS on the development and maintenance of hypertension in this model. This will have to be accomplished in triple transgenic mice that endogenously express the cre-recombinase in the liver. Studies using the R 

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-12lox in conjunction with albumin promoter cre-recombinase (and other cell-specific promoters) transgenic mice are already in progress.

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