

Efficient Liver-specific Deletion of a Floxed Human Angiotensinogen Transgene by Adenoviral Delivery of Cre Recombinase *in Vivo**

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Tissue-specific ablation of gene function is possible *in vivo* by the Cre-loxP recombinase system. We generated transgenic mice containing a human angiotensinogen gene flanked by loxP sites (hAGT^{lox}). To examine the physiologic consequences of tissue-specific loss of angiotensinogen gene function *in vivo*, we constructed an adenovirus expressing Cre recombinase. Studies were performed in several independent lines of hAGT^{lox} mice before and after intravenous administration of either Adcre or Ad β Gal as a control. Systemic administration of Adcre caused a significant decrease in circulating human angiotensinogen and markedly blunted the pressor response to administration of purified recombinant human renin. Southern blot analysis of genomic DNA from various organs revealed that the Cre-mediated deletion was liver-specific. Further analysis revealed the absence of full-length human angiotensinogen mRNA and protein in the liver but not the kidney of Adcre mice, consistent with the liver being the target for adenoviruses administered intravenously. These studies demonstrate that extra-hepatic sources of angiotensinogen do not contribute significantly to the circulating pool of angiotensinogen and provide proof-of-principle that the Cre-loxP system can be used effectively to examine the contribution of the systemic and tissue renin-angiotensin system to normal and pathological regulation of blood pressure.

The renin-angiotensin system (RAS)¹ has long been known to play a major role in the regulation of blood pressure and extracellular fluid volume through the systemic and intrarenal actions of angiotensin II. Classically, angiotensin II is processed in the circulation by the successive proteolytic actions of renin and angiotensin-converting enzyme on angiotensinogen

(AGT). Circulating AGT is derived mainly from the liver, although it is also synthesized by the kidney, heart, brain, vasculature, and fat (1). Although extra-hepatic AGT is not thought to provide a source of circulating AGT, local synthesis of other components of the RAS including angiotensin II receptors in these tissues suggests the potential for both local synthesis and action of angiotensin II. Indeed, angiotensin II has been postulated to regulate blood flow, natriuresis, and tubuloglomerular feedback in the kidney (2, 3) and to facilitate neurotransmission, sympathetic outflow, and arginine vasopressin release in the central nervous system (4, 5). Angiotensin II may also play an important role in the development of some organs (6). Therefore, elucidating the function of local RAS synthesis has been of substantial interest to many investigators. Unfortunately, these studies have been complicated by the absence of experimental tools to distinguish these tissue-based systems from the classical systemic (blood-borne) or endocrine system.

Recent advances in genetic techniques, which allow gene ablation and tissue-specific gene targeting may provide an important new set of tools to experimentally dissect tissue-based regulatory circuits such as the RAS. Tissue-specific inactivation of a gene would be particularly useful if the gene is expressed in a wide variety of tissues, is part of a complex paracrine system, or whose global loss of function results in death or severe organ dysfunction. Gene-targeted disruption of genes known to be important in blood pressure regulation, such as AGT, angiotensin-converting enzyme, angiotensin II receptors, and endothelin, lead to malformations, organ dysfunction, or death, thus impeding studies on blood pressure regulation (7–10). The use of the Cre-loxP recombinase system may afford a solution to this problem by providing a tool to induce knock-outs of a gene in a specific tissue and under temporal control (11, 12). To accomplish this, a gene is targeted (“floxed”) with loxP sites, which are 34-base pair palindromic sequences, which each bind two molecules of Cre recombinase. Cre recombinase can catalyze a homologous recombination event between two loxP sites found in the same orientation on a contiguous DNA molecule, causing a deletion of the intervening DNA sequence. Genes that are appropriately modified to contain loxP sites in introns flanking an important coding exon will be functional in the absence of Cre recombinase, because the loxP site will be spliced out during mRNA processing but will be rendered nonfunctional in the presence of the Cre recombinase (reviewed in Ref. 13). Because the floxed gene will be found in every cell, the specificity of the system arises from tissue-specific delivery of Cre recombinase. This can be achieved either by its expression as a transgene under the control of a highly tissue-specific promoter (14) or delivered specifically to a tissue with the use of a viral vector (15–17). We demonstrate

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¹ The abbreviations used are: RAS, renin-angiotensin system; AGT, angiotensinogen; PCR, polymerase chain reaction; kb, kilobase(s); Ad, adenovirus.

herein efficient liver-specific deletion of a floxed gene after adenoviral delivery of Cre recombinase and report its effects on plasma AGT and blood pressure.

MATERIALS AND METHODS

Generation of Transgenic Mice—The transgene used herein containing the human AGT (*hAGT*) gene is identical to one previously described in detail except for the presence of loxP sites flanking exon 2 (Fig. 1) (18–20). The *hAGT* gene was digested with *Bam*HI to remove the entire portion of exon 2 along with flanking sequences to form the plasmid hAGT Δ Bam. This plasmid was then ligated to loxP oligonucleotides consisting of two loxP sites separated by an internal *Bgl*II site with *Bam*HI ends. The resulting plasmid (hAGT Δ Bam^{lox}) was digested with *Bgl*II and religated to the exon 2 *Bam*HI fragment to yield the final hAGT^{lox} construct. All plasmids were sequenced to confirm the orientation of the loxP sites, and their function was first tested by restriction digestion of plasmid DNA grown in a bacterial strain (BS591) constitutively expressing Cre recombinase (21). The transgene segment was obtained by digestion with *Nhe*I and separated by agarose gel electrophoresis. The DNA fragment was recovered using the Qia-Quick purification kit (Qiagen) and was microinjected into fertilized C57BL/6J \times SJL/J (B6SJL F2) mouse embryos as described previously (18). Care of the mice used in the experiments met or exceeded the standards set forth by the National Institutes of Health in their *Guidelines for the Care and Use of Experimental Animals*. All procedures were approved by the University Animal Care and Use Committee at the University of Iowa. All mice were fed standard mouse chow (LM-485; Teklad Premier Laboratory Diets, Madison, WI) and water *ad libitum*.

Construction and Delivery of Adenoviruses—The Cre recombinase expression cassette containing the human CMV promoter and metallothionein polyadenylation signal was subcloned from pBS185 generously provided by Dr. Brian Sauer into the adenovirus shuttle plasmid (22). This shuttle plasmid and the Ad5 backbone, sub360, were co-transfected into HEK293 cells. Adcre was purified using conventional techniques (23, 24) by the University of Iowa Gene Transfer Vector Core. Cell lysates from infected cells were evaluated for Cre recombinase activity *in vitro* as described (25). Site-specific recombination was confirmed by PCR analysis and sequencing. Recombinant Adcre were plaque-purified and concentrated using CsCl centrifugation. Ad β gal has been previously described in detail elsewhere (26, 27).

Experiments were performed on 8- to 10-week-old mice. Adenoviruses at a concentration of 1.1×10^{12} plaque-forming units/ml (in 0.1 ml) were administered intravenously via tail vein injection or direct cannulation of the right jugular vein. For implantation of the jugular vein catheter, mice were anesthetized with sodium pentobarbital (50 mg/kg), and a catheter (PE10) was inserted into the right jugular vein. Following the delivery of adenovirus, the catheter was trimmed, sealed, and enclosed inside the mouse. For mice in which the pressor response to infused human renin was tested, the catheter was tunneled subcutaneously and exteriorized between the scapulae as described (28). No differences in the overall efficiency or tissue specificity of infection were observed between the two methods of delivery (data not shown).

Measurement of Blood Pressure—Blood pressure was measured in conscious, freely moving mice surgically instrumented with a left common carotid artery catheter, and infusions of purified recombinant human renin were made via a catheter implanted into the right jugular vein as described (28). Catheters were tunneled subcutaneously, exteriorized between the scapulae, and were filled and flushed daily with sterile dilute heparinized saline (50 units/ml). Mean arterial blood pressure was measured on a Grass Model 7 polygraph using Cobe transducers. Data was acquired on a Gateway 2000 model E-3000 computer running the Polyview Software provided by Grass Instruments. After a 15-min stabilization period, human renin (500 ng or 1 μ g in a 10- μ l volume, the gift of Drs. Walter Fischli and Klaus Lindpaintner at F. Hoffman-LaRoche, Basel Switzerland) was infused while blood pressure was continuously recorded. Mice in which infusion of purified human renin did not elicit a pressor response after 15 min were then given an infusion of a known pressor dose of angiotensin II (10 mg/kg).

Analysis of Nucleic Acids—Genomic DNA was purified from tail biopsies and subjected to Southern blot or PCR analysis as described (18). To determine tissue-specific recombination, genomic DNA was isolated from various tissues from adenovirus-infected mice using the proteinase K-phenol extraction protocol (29). For Southern blot analysis, 10 μ g of purified genomic DNA was digested with *Eco*RI and separated on 0.8% agarose gel, transferred to nitrocellulose membrane, and hybridized with a radioactively labeled probe derived from either intron 3 or exon 2 of the *hAGT* gene. The probe detects a 12.5-kb

wild-type band and a 7-kb Cre-mediated deletion band. Total tissue RNAs were isolated by a modified guanidine isothiocyanate method as described previously (18). Tissue-specific expression studies were performed by Northern blot analysis using a hAGT cDNA probe derived from exon 2 (18). A mouse β -actin probe was used to determine the integrity of purified RNA and the equal loading of samples (pTRI-actin, Ambion).

Plasma Angiotensinogen Assay—Mouse and human AGT can be differentiated in the plasma on the basis of the strict species specificity of the biochemical reaction between renin and AGT (18, 30). Plasma samples were obtained daily by orbital eye bleed from Adcre and Ad β gal mice except for groups in which the pressor response to infused human renin was studied. Mice in those groups were bled at the start of the experiment and then again on day 5 and day 7 post-infection, before the infusion of human renin. Approximately 125 μ l of whole blood was collected at each bleed and placed in chilled tubes containing 2.5 μ l of 0.5 mol/liter EDTA. The specimens were then immediately centrifuged at 12,000 rpm for 2 min at 2 $^{\circ}$ C, and a 50- μ l plasma sample was obtained and immediately frozen at -80° C. Plasma samples were treated as described previously (18, 31). Radioimmunoassays were then performed using the RIANEN angiotensin I ¹²⁵I-labeled radioimmunoassay kit (Dupont) using the directions and reagents supplied by the manufacturer. Samples were appropriately diluted with reagent blank so that the radioimmunoassay results were on the linear portion of the standard curve. Plasma levels of AGT were then extrapolated using the 1:1 molar relationship between Ang-I and its precursor, AGT, using the formula ng of Ang-I/ml \times 0.77 pmol/ng Ang-I \times 0.05 μ g of hAGT/pmol.

Immunofluorescent Staining and Immunoblots—Liver and kidney samples were removed immediately after mice were killed and placed in 4% paraformaldehyde solution on ice for 2 h. Tissue samples were then rinsed in ice-cold PBS and prepared for cryo-sectioning using standard techniques. Processed tissues were stored at -80° C in O.C.T. compound until use. Tissue sections (10- μ m thick) were permeabilized with 0.1% Triton X-100 in Superblock (Pierce) for 10 min at room temperature and incubated 16–18 h with a polyclonal antibody against hAGT, kindly provided by Dr. Duane Tewkbury, Marshfield Medical Research Foundation (1:5,000 dilution) at 4 $^{\circ}$ C. Samples were washed in PBS and exposed to CY3-labeled donkey anti-rabbit IgG (1:500 dilution) for 2 h at 37 $^{\circ}$ C. Images were obtained using fluorescence confocal microscopy and were captured digitally.

Plasma samples (3 μ l) were run on a 10% SDS-acrylamide gel, transferred electrophoretically to nitrocellulose, and probed with the same polyclonal antibody as above (1:10,000 dilution). Protein bands were detected using a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Opti-4CN substrate kit, Bio-Rad) using directions provided by the manufacturer.

Statistics—All numerical data are presented as the mean \pm S.E. Statistical analysis was performed using Student's *t* test using the SigmaPlot software package or by ANOVA using the Systat software package.

RESULTS

To appraise the utility of the Cre-loxP recombinase system to examine the significance of tissue AGT expression and to assess the efficiency of liver-specific deletion of AGT, we designed a transgene (*hAGT*^{lox}) in which exon 2 of the *hAGT* gene is flanked by loxP sites (Fig. 1). Exon 2 contains the angiotensin precursor peptide sequences and a majority of the protein coding region of the gene, and thus its elimination upon exposure to Cre recombinase will render the gene nonfunctional for the production of hAGT or angiotensin II. However, a normal hAGT mRNA should be transcribed in cells lacking Cre recombinase because the loxP sites were inserted into introns and will be spliced out during mRNA processing.

Four lines of hAGT^{lox} mice were initially established that exhibited widely varying levels of plasma hAGT protein, due likely to differences in both transgene copy number and integration site (Table I). We limited further experimentation to lines 4258/1 and 4284/1, which express hAGT at a low (10.5 ± 1.6 μ g/ml) and moderate (237 ± 17.5 μ g/ml) level, respectively. Lines 4331/1 and 4252/1 were eliminated from further analysis as the level of plasma hAGT was much higher than that found in normal human plasma (32). The tissue-specific pattern of expression of the hAGT^{lox} transgene in both lines was similar

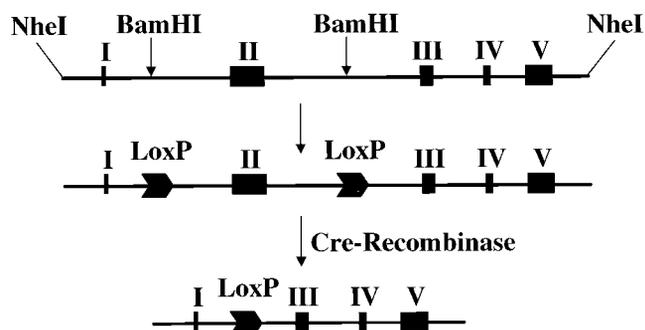


FIG. 1. **Schematic map of the transgene.** A schematic representation of the transgene is shown. The *top map* shows the original hAGT transgene showing the location of *Bam*HI sites. The *middle map* shows the replacement of these *Bam*HI sites with loxP sites (*arrowheads*). The *bottom map* shows a representative transgene after Cre-mediated recombination. Cre recombinase causes a homologous recombination between the two loxP sites, resulting in the deletion of the intervening DNA and retention of a single loxP site.

TABLE I
Plasma hAGT in hAGT^{loxP} mice

Plasma hAGT was examined in each of the indicated lines and is represented as both ng of angiotensin I converted/ml in the assay and was also extrapolated to μ g of hAGT/ml as described under "Materials and Methods."

Transgenic line	Plasma human angiotensinogen		N
	ng angiotensin I converted/ml	μ g/ml	
4258/1	274 \pm 42	10.5 \pm 1.6	20
4284/1	6,203 \pm 457	237 \pm 17.5	19
4331/1	15,086 \pm 1,100	577 \pm 42.1	10
4252/1	125,275	4,795	1

to the expression of hAGT transgenes lacking loxP sites (Fig. 2) (18). The highest level of expression was observed in liver and kidney with much lower level expression in a number of extrarenal and extra-hepatic tissues. In addition, as previously reported for hAGT transgenes lacking loxP sites, expression of hAGT in the kidney was higher in males than in females.

To assess the overall efficiency of Cre-loxP-mediated deletion of hAGT^{loxP}, we first measured circulating hAGT protein before and after intravenous administration of an adenovirus containing either Cre recombinase (Adcre) or β -galactosidase (Ad β gal). This is an ideal model system to examine the efficiency of the system as adenoviruses administered intravenously mainly infect the liver, which is the major source of circulating hAGT (33). Western blot analysis revealed a significant decrease in circulating hAGT 5-days after administration of Adcre but no change after administration of the control Ad β gal virus (Fig. 3A). We examined the time course of Cre-mediated hAGT^{loxP} deletion by obtaining plasma samples before and each day after adenovirus administration and employed a biochemical assay to accurately measure hAGT after its complete conversion by human renin to Ang-I (18). Administration of the Ad β gal adenovirus had no significant effect on the levels of circulating hAGT in the plasma of either transgenic line (Fig. 3, B and C). However, a decrease in circulating hAGT was evident 1 day after Adcre administration, and levels continued to decline through day 3 in line 4258/1 (Fig. 3C) and through day 5 in line 4284/1 (Fig. 3B). There was essentially no circulating hAGT in line 4258/1 after 3 days of Adcre infection, as 2 μ g/ml represents the background of the assay under these conditions. Circulating hAGT in line 4284/1 fell by approximately 90% from 180.2 μ g/ml to 19.4 μ g/ml after 5 days of Adcre. Neither Adcre or Ad β gal had any effect in transgenic mice containing a hAGT construct lacking loxP sites (data not shown).

Northern blots examined with an exon 2-specific probe (in-

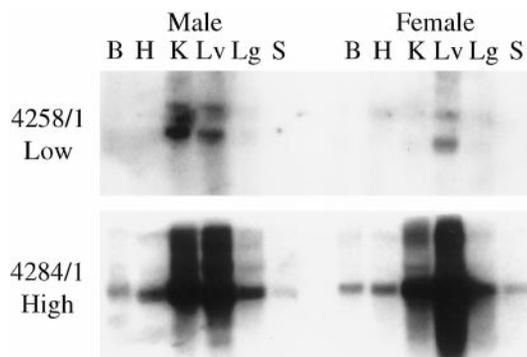


FIG. 2. **Tissue-specific expression of hAGT^{loxP}.** Northern blot analysis of hAGT mRNA from tissues obtained from two lines of hAGT^{loxP} mice is shown. One representative blot from one male and one female of each line is shown. No expression was detected in any non-transgenic control mice. B, brain; H, heart; K, kidney; Lv, liver; Lg, lung; S, spleen.

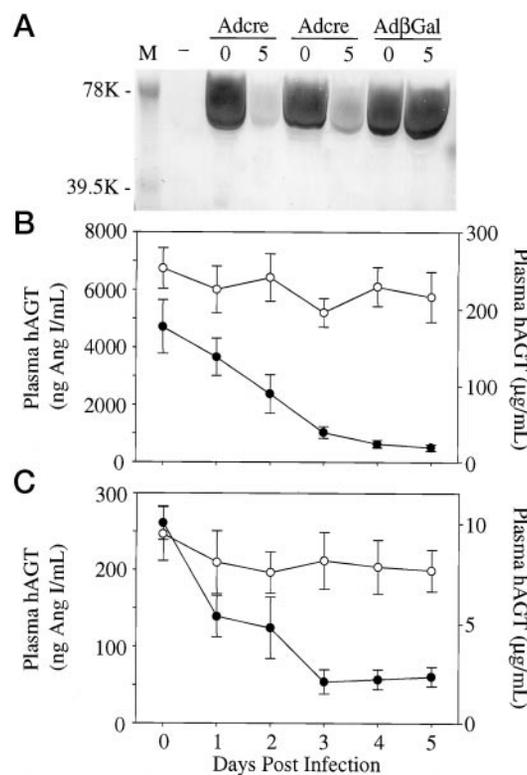


FIG. 3. **Plasma hAGT after Adcre and Ad β gal infection.** A, Western blot analysis of 3 μ l of total plasma protein obtained from mice before (0) and 5 days post-infection. Plasma samples from two different Adcre-infected mice, one Ad β gal-infected mouse, and one uninfected nontransgenic control (-) are shown. Molecular weight markers are indicated. B and C, plasma hAGT was determined by its complete conversion by human renin to Ang-I followed by radioimmunoassay as described under "Materials and Methods." Samples were obtained from orbital bleed before (0) and each day for 5 days after infection with Ad β gal (\circ) or Adcre (\bullet) from line 4284/1 ($n = 6$, Adcre and Ad β gal, panel B) and 4258/1 ($n = 5$ for Adcre, $n = 7$ for Ad β gal, panel C).

ternal to the deletion) revealed a marked decrease in hAGT mRNA in the liver of Adcre-infected mice but not Ad β gal-infected mice, consistent with hAGT levels in plasma (Fig. 4). Northern blots using an exon 5-specific probe lying outside of the deleted region revealed the presence of a truncated hAGT mRNA in liver of Adcre mice, and reverse transcription-PCR followed by sequence analysis indicated that this resulted from an accurate splicing of exon 1 to exon 3 (data not shown). The tissue specificity of Cre-mediated recombination was validated by the unaltered level of hAGT mRNA in the kidney of both

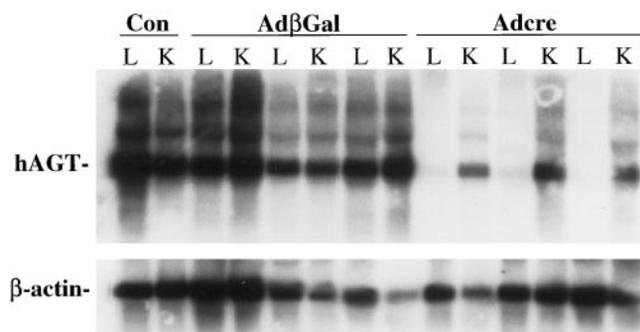


FIG. 4. **hAGT mRNA in kidney and liver of Adcre and Adβgal-infected mice.** Northern blot analysis of liver (L) and kidney (K) mRNA from uninfected (Con) and Adβgal- and Adcre-infected mice 5 days post-infection. Each pair of samples is from a different mouse from the 4284/1 transgenic line. The *upper blot* is probed for hAGT expression, and the *lower blot* is probed for β-actin expression as indicated. There was no expression of hAGT in any nontransgenic mice examined.

Adcre- and Adβgal-infected mice. We were also unable to detect any mRNA arising from a Cre recombinase-mediated deletion of *hAGT^{fllox}* gene in the kidney of Adcre-infected mice using reverse transcription-PCR (data not shown).

We assayed for hAGT protein in liver sections from line 4284/1 by immunofluorescence using a polyclonal antibody specific for hAGT. Only background staining was observed in nontransgenic mice or in sections lacking primary antibody (data not shown). High levels of hAGT were detected uniformly throughout the liver in mice infected with the Adβgal virus (Fig. 5, *left panel*), but only background staining and occasional small patches of hAGT-positive cells were detected in liver from Adcre-treated mice (Fig. 5, *right panel*). This data along with the analysis of plasma hAGT and hepatic hAGT mRNA strongly suggests that the overall efficiency of Cre-mediated *hAGT* gene deletion exceeded 90%.

The tissue specificity of intravenously administered Adcre was further validated by Southern blot analysis. Preliminary analysis suggested that the transgene copy number in each line was between 5 and 10. Genomic DNA was isolated from several different organs of a 4258/1 line mouse and an approximately 7.0-kb band representing Cre-mediated recombination was evident in the liver (Fig. 6). There was no evidence of recombination in brain, kidney, lung, or spleen, confirming that Adcre administration can specifically target recombination in the liver. The 7.0-kb band was positively identified as the Cre-mediated deletion product, as it was not detected when the Southern blots were reprobed with an exon 2-specific (internal to the deletion) probe (data not shown). Despite the very efficient elimination of hepatic hAGT mRNA and circulating hAGT, the extent of recombination detected by Southern analysis was reproducibly observed to be between 50 and 75% in representative mice from both lines (data not shown).

Finally, we examined the physiological significance of the decreased levels of circulating hAGT in *hAGT^{fllox}* mice by measuring the pressor response to an infusion of purified recombinant human renin. Pressor responses were measured in both transgenic lines under control conditions or after infection with either Adcre or Adβgal. There is a strict species specificity in the enzymatic reaction between renin and AGT (30). Therefore, human renin will only cause a pressor response if hAGT is also circulating simultaneously (18). Dose-response curves were first performed in both the 4284/1 and 4258/1 transgenic lines to obtain a dose of purified human renin that would elicit a significant pressor response. Under control (uninfected) conditions, infusion of 0.5 μg of purified recombinant human renin elicited a 30 mm of Hg increase in blood pressure in 4284/1 mice (Fig. 7A). In 4258/1 mice, 1 μg of purified recombinant

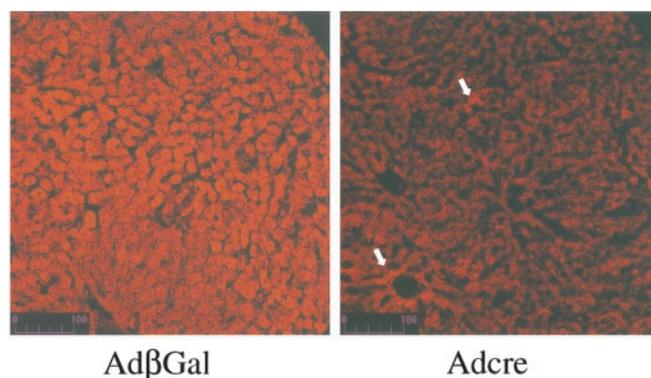


FIG. 5. **Localization of hAGT in Adβgal- and Adcre-infected liver.** Frozen liver sections were cut, and hAGT protein was detected as described under "Materials and Methods." Liver sections were derived from Adβgal (*left panel*) or Adcre (*right panel*) mice as indicated. The *arrowheads* point to pockets of hAGT-positive cells. The scale represents 100 μm.

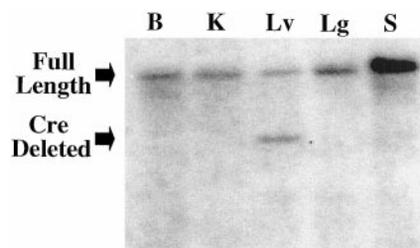


FIG. 6. **Cre-mediated deletion of *hAGT^{fllox}* at the DNA level.** Southern blot analysis of genomic DNA isolated from brain (B), kidney (K), liver (Lv), lung (Lg), and spleen (S) is shown. 10 μg of genomic DNA was treated as described under "Materials and Methods." *EcoRI* digestion results in bands of 12.5 kb for full-length and 7.0 kb for Cre-deleted.

human renin was found to be necessary to elicit the same response. Both of these doses of purified human renin had no effect on blood pressure when administered to nontransgenic mice (data not shown). Infusions of purified human renin were then performed in separate groups of transgenic mice 5 days post-infection with Adcre or Adβgal (Fig. 7, B–D). Pressor responses to human renin were unchanged 5 days after Adβgal infection (data not shown). Two response patterns were noted in the Adcre-treated group: 1) those that exhibited a pressor response of normal magnitude but of very short duration (Fig. 7B) and 2) those that exhibited no pressor response (Fig. 7C). All mice exhibited a normal pressor response to an infusion of angiotensin II, indicating the mice did not lose the ability to respond to angiotensin II (Fig. 7D). When analyzed as a group, human renin elicited a pretreatment 31.2 ± 2.7 and 25.6 ± 2.7 mm of Hg pressor response in lines 4284/1 and 4258/1, respectively, which lasted for nearly 60 min before returning to base line (Fig. 8). Five days after Adcre, the pressor response decreased to 20.5 ± 6.0 and 11.5 ± 4.5 mm of Hg and lasted only 20.0 ± 4.0 and 14.4 ± 6.5 min for the two lines, respectively. Because some mice still exhibited a pressor response after 5 days of treatment, the human renin infusion was repeated in some of these mice on day 7. There was essentially no pressor response in either line 7 days after Adcre treatment (Fig. 8).

DISCUSSION

The RAS is a complicated regulatory system which has been proposed to coordinate a variety of diverse physiological functions including blood pressure, electrolyte balance, blood flow, thirst, inflammation, reproduction, neural transmission, cellular growth, and organ development. Abnormalities in the system have been proposed to cause hypertension, cardiac hypertrophy, renal fibrogenesis, and may have adverse effects on

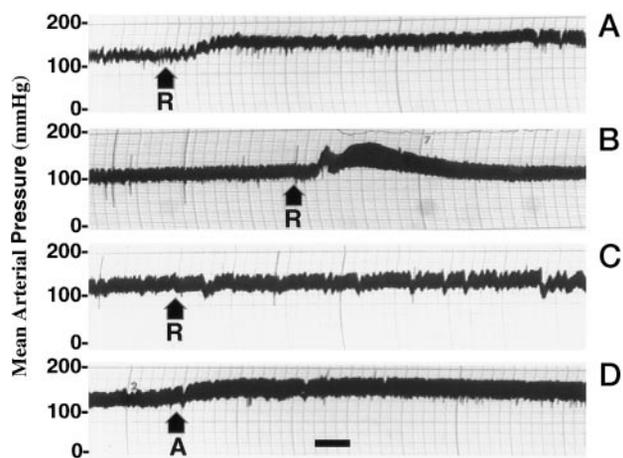


FIG. 7. **Typical blood pressure tracings.** Four blood pressure tracings illustrating typical responses to an infusion of human renin (panels A-C) or angiotensin II (panel D) are shown. All tracings are derived from transgenic line 4284/1 mice. A, sustained pressor response in a transgenic mouse before adenoviral administration; B, transient pressor response 5 days after Adcre infection; C, no pressor response 5 or 7 days after Adcre infection; D, sustained pressor response to an infusion of angiotensin II. Arrows indicate time of human renin (R) or angiotensin II (A) infusion. The bar in panel D represents 1 min.

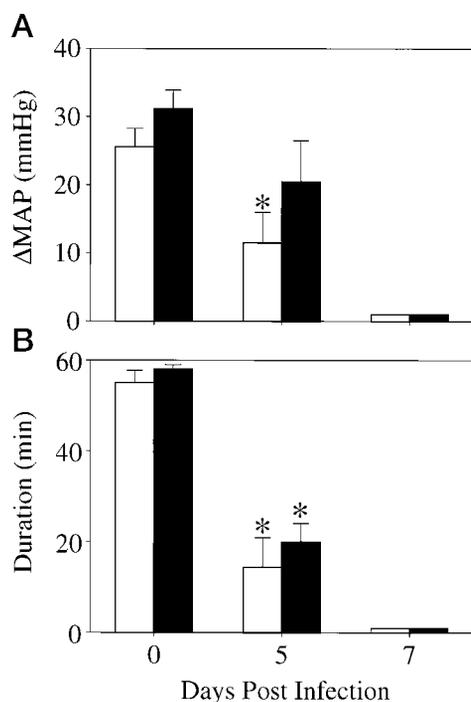


FIG. 8. **Summary of blood pressure data.** The peak change in mean arterial pressure (A) and duration of the pressor response (B) are shown 0, 5, and 7 days after Adcre infection. Open bars, line 4258/1; closed bars, line 4284/1. *, $p < 0.02$ in A, and $p < 0.0002$ in B versus 0 time point. Line 4258/1, $n = 8$ for day 0, $n = 10$ for day 5, and $n = 5$ for day 7. Line 4284/1, $n = 6$ for day 0, $n = 10$ for day 5, and $n = 2$ for day 7.

reproduction and organ development. Experimentally, our understanding of the system is complicated by the finding that RAS genes are expressed in many tissues, some of which may have the ability to locally synthesize and respond to angiotensin II. Much of what we have learned regarding the physiological importance of the RAS was gained from studies examining its tissue-specific expression and response to local or systemic infusion of angiotensin II or its inhibitors. Indeed, these studies are limited by the specificity of the inhibitors employed and by the difficulty in achieving their local delivery and retention.

Although knockout mice generated by gene-targeting in embryonic stem cells have provided important information on the function of the RAS, these studies are limited because the gene is eliminated from all tissues, and as a result, these animals exhibit premature death or severe organ dysfunction that precludes a detailed analysis of cardiovascular function (9, 34, 35). Similarly, although transgenic models containing the RAS genes have provided definitive evidence that overexpression of the system causes hypertension, the models have not been sufficient to address the organ- and cell-specific mechanisms involved (36;37). For example, although recent molecular, genetic, and pharmacologic studies suggest that the hypertension in double transgenic mice containing the human renin and *hAGT* genes may be mediated by the synthesis and action of angiotensin II in the systemic circulation, kidney, and central nervous system, the relative contributions of the different systems remains unclear (28, 37, 38).

The long term goal of these studies is to develop an experimental system to assess the importance of tissue-specific expression of AGT in the regulation of blood pressure and electrolyte balance by experimentally separating systemic from tissue RAS systems. As a first step toward accomplishing this goal, we combined the use of transgenic expression of a floxed *hAGT* construct with adenoviral delivery of Cre recombinase. We considered this to be an attractive approach for two reasons. First, the liver is thought to be the primary site of AGT synthesis and source of circulating AGT. Elimination of hepatic AGT should therefore deplete the circulating store of AGT without affecting secondary sites of synthesis such as the kidney, heart, or brain. Second, previous studies have demonstrated that intravenous administration of adenoviruses efficiently infects the liver but not other tissues (33). Adenoviral delivery of Cre recombinase should provide the liver specificity needed for the generation of a liver-specific knockout without the need for the generation of a transgenic mouse expressing Cre recombinase under the control of a hepatic promoter. Moreover, adenoviral delivery of Cre recombinase provides temporal control over when the deletion is generated, which would otherwise require the use of an inducible/repressible promoter.

The only potential drawback from this approach is the well known inflammatory response exhibited after adenoviral infection. An enlarged spleen, indicative of an inflammatory response, was noted in all Ad-treated mice, and liver inflammation is routinely observed after adenoviral infection. Clearly, this did not influence the results of our experiments, as inflammation was observed in both the Adcre and Ad β gal mice, whereas the reduction in *hAGT* was noted only in Adcre mice. Methods designed to reduce inflammation and to allow repeated dosing of adenoviruses have been reported and may be applicable to these types of studies (39, 40).

We demonstrated that the *hAGT^{fllox}* transgene exhibited the same pattern of tissue-specific expression as a transgene lacking loxP sites, and the level of transgene expression was proportional to copy number as previously reported (18, 19). Moreover, the liver specificity of the Cre-mediated deletion was confirmed by both Southern and Northern blot hybridization analysis. The deletion of hepatic *hAGT* mRNA resulted in a 90–100% decrease in circulating *hAGT* in both lines of mice and occurred despite the presence of abundant *hAGT* mRNA in the kidney and other tissues of Adcre-infected mice. These data suggest that most, if not all the AGT in the systemic circulation is derived from the liver and that AGT locally synthesized in extra-hepatic tissues is not released into the circulation. This hypothesis is supported by evidence from the cell-specific targeting of *hAGT* to renal proximal tubule cells, demonstrating that *hAGT* locally synthesized in the kidney is not released into

the systemic circulation (38). This observation is also supported by the blunted pressor response to human renin infusion exhibited by Adcre-infected mice 5-days post-infection. As indicated above, some mice failed to exhibit a pressor response, whereas others exhibited a pressor response that was greatly reduced in duration. When the human renin infusion was repeated 2 days later, none of the mice exhibited a pressor response to the same dose of human renin. This finding suggests that the residual hAGT in plasma may have been cleared by the enzymatic activity of human renin. This absence of bioavailable hAGT in the plasma 2 days after human renin infusion further suggests that extra-hepatic tissues do not make a significant contribution to circulating AGT.

The overall efficiency of the Cre-loxP recombinase system will be dependent upon the level of Cre recombinase expressed within a cell and the number of cells expressing Cre recombinase. In these experiments this should be directly proportional to the number of cells infected with the Adcre virus. Consequently, it is not surprising that the deletion of the *hAGT^{fllox}* gene is a stochastic process, which may not be 100% efficient, and this is consistent with the identification of clusters of hAGT-positive cells in the liver of Cre-treated mice. One aspect of these experiments that remains unclear, however, is the presence of significant levels of full-length (unaltered) hAGT^{fllox} transgene in genomic DNA from the liver. An analysis of liver genomic DNA from several different animals and transgenic lines consistently revealed that 50–75% of the total hAGT^{fllox} DNA was converted to the defective form by Cre recombinase. This may reflect the fact that the transgene copy number in each line was approximately 5–10. Therefore there may be incomplete transgenes at the termini of the integrated array, both “head-to-head” and “head-to-tail” arrangements of the transgenes, or rearrangements of the transgene segment in some cells, which make the observed efficiency of recombination (by Southern) lower than that suggested by the expression data. Nevertheless, despite the presence of full-length transgene DNA, we observed a nearly complete depletion of hAGT mRNA in this tissue. In fact, the data obtained using the exon 2-specific probe, which lies internal to the deleted region (Fig. 4), was also confirmed with an exon 5-specific probe, which lies in a portion of the mRNA that would be retained after the Cre-mediated deletion (data not shown). The exon 5-specific probe did not detect any full-length mRNA but instead detected a truncated hAGT mRNA that reverse transcription-PCR revealed was derived from a splicing of exon 1 to exon 3. Although it is difficult to reconcile the differences in Cre recombinase efficiency assessed at the DNA and mRNA level, it remains clear that Adcre delivery was efficient in eliminating hAGT protein from the plasma. Therefore, our results clearly demonstrate that adenoviral delivery of Cre recombinase is an effective tool for the generation of a liver-specific knockout of a floxed gene. Moreover, these results provide an important proof-of-principle that the Cre-loxP system can be used effectively to study the importance of tissue-specific RAS in cardiovascular physiology.

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