Androgen-dependent regulation of human angiotensinogen expression in KAP-hAGT transgenic mice

YUEMING DING AND CURT D. SIGMUND
Genetics Interdisciplinary Graduate Program, Departments of Internal Medicine and Physiology and Biophysics, The University of Iowa College of Medicine, Iowa City, Iowa 52242

Ding, Yueming, and Curt D. Sigmund. Androgen-dependent regulation of human angiotensinogen expression in KAP-hAGT transgenic mice. Am J Physiol Renal Physiol 280: F54–F60, 2001.—We previously reported a novel transgenic model expressing human angiotensinogen from the kidney androgen-regulated protein promoter, and demonstrated sexually dimorphic expression. Herein, we investigated the hormonal regulation of this transgene. Testosterone increased transgene expression in female mice in a dose- and time-dependent manner and was not detectable 3-days after treatment was halted. High doses of estrogen were required to induce the transgene. Expression of transgene mRNA decreased after castration of male transgenic mice. As in males, however, transgene expression could be induced after administration of testosterone. Flutamide, an androgen receptor antagonist, dose dependently blocked transgene expression in males and blunted the induction caused by testosterone in females. Neither testosterone nor estrogen altered the proximal tubule cell-specific expression of the transgene. The data suggest that the level of transgene expression in this model can be controlled temporally and in magnitude by manipulating the levels of androgen. The fortuitous androgen regulation of this transgene can be used as a molecular “on-off” switch to control transgene expression and potentially manipulate blood pressure levels in this model.

renin-angiotensin system; hormonal regulation; inducible gene expression; hypertension

ANGIOTENSINOGEN (AGT) has been shown to be modulated by many factors such as glucocorticoids, estrogens, androgens, thyroid hormone, cytokines, and ANG II (8, 19). These hormones can stimulate synthesis of AGT in cultured cell lines, animal models, and humans (6, 10, 17, 18), and this induction is tissue specific. For instance, androgen has been reported to increase kidney AGT mRNA levels, whereas it has only a slight effect on liver AGT mRNA levels (11). Similarly, estrogens can induce AGT expression in both kidney and liver but not in aorta and adipose tissue (4, 13). The induction of AGT by these hormones can be neutralized by antagonists such as anti-glucocorticoid and anti-estrogen reagents, or by surgical treatments such as adrenalectomy and thyroidectomy (2, 29). Most of the hormones are believed to regulate AGT expression at the transcriptional level because their actions occur rapidly and can be blocked by actinomycin D, a specific inhibitor of transcription. Indeed, several hormonal response elements such as glucocorticoid response element (GRE), estrogen response element (ERE), thyroid hormone response element (TRE), and acute-phase response element (APRE) have been identified in the 5′ flanking region of the human, rat, and mouse AGT genes (3). One of the GREs in both the human and rat gene is essential for glucocorticoid induction, and the other GRE can synergize this effect (12).

Like AGT, the kidney androgen-regulated protein (KAP) gene is regulated by multiple hormones. The KAP gene encodes a protein of unknown function and was initially identified by its detection as a very abundant androgen-regulated mRNA in kidney (34). Serial analysis of gene expression has revealed that KAP is the second-most abundant mRNA in kidney (35). It is expressed specifically in the epithelial cells of the proximal tubules although different regions of the proximal tubule appear differentially responsive to steroid hormones (5, 22, 36). For example, androgens regulate the expression of KAP mRNA in epithelial cells of the S1, S2, and S3 segments of the proximal tubule, whereas estrogen and thyroid hormone control the expression of KAP mRNA primarily in the S3 segment (23, 32, 33). In female congenital thyroid hormone-deficient hyt/hyt mice, there is no KAP mRNA expression, suggesting that thyroid hormones are required for its expression in the kidney of females (32, 33). Males and testosterone-induced females express KAP mRNA throughout the entire proximal tubule. In contrast, females, castrated males, and androgen receptor-deficient Tfm/Y mutant mice show KAP mRNA exclusively in the S3 segment of the proximal tubule (24, 25). Similar to AGT, several hormone-response elements such as ERE, TRE, and androgen response element (ARE) have also been identified in the 5′ flanking region of the KAP gene (28).

Transgenic mice containing the human angiotensinogen (hAGT) gene driven by the KAP promoter (KAP-hAGT) exhibit sexually dimorphic expression of the transgene specifically in renal proximal tubule cells (9). In male transgenic mice, renal expression of
the transgene is constitutively active. In contrast, in female transgenic mice, KAP-hAGT mRNA was undetectable under baseline conditions but could be markedly induced by administration of testosterone. The purpose of the present study was to assess the ease with which the KAP promoter could be hormonally regulated in male and female KAP-hAGT transgenic mice. KAP-hAGT expression was examined in females treated with testosterone or estrogen, and in males treated with the androgen-receptor antagonist flutamide or after castration. Because double transgenic mice containing the human renin and KAP-hAGT transgenes are hypertensive, our long-term goal of the present study is to develop a “molecular switch” to control the blood pressure of hREN/KAP-hAGT mice by manipulating the levels of KAP-hAGT expression (7).

**METHODS**

Transgenic mice and animal husbandry. KAP-hAGT single transgenic mice were generated and characterized as previously described (9). All mice were maintained by backcross breeding to C57BL/6J mice. Transgenic mice were identified by PCR amplification of DNA isolated from tail biopsies as described previously (9). All mice were fed standard mouse chow (Teklad LM-485) and water ad libitum unless otherwise indicated. Care of mice met or exceeded the standards set forth by the National Institutes of Health in the Guidelines for the Care and Use of Laboratory Animals (NIH publication 86–23, revised 1985). All procedures were approved by the University of Iowa Animal Care and Use Committee. Experimental mice were killed by CO₂ asphyxiation.

Administration of drugs and surgery. All experiments were performed in duplicate. Representative results are shown. Female mice were treated with testosterone or estrogen by administration of a testosterone or estrogen pellet. The testosterone pellet (catalog no. A-151, Innovative Research of Sarasota, FL) contains 5 mg testosterone continuously released for 21 days. Different dosages of estrogen pellets (catalog no. A-121, Innovative Research of America) were used: 1, 5, 10, 25, 75, and 200 mg. They were also designed for continuous release for 21 days. Mice were first anesthetized with metofane (0.1 ml in an inhalation chamber), and the pellet was implanted subcutaneously in the back and tunneled to the nape of the neck with a 10-gauge trocar. The incision was sutured, and the mice were allowed to recover on a heating pad. The whole procedure took <5 min. Mice treated with a testosterone pellet or sham-operated control mice were killed at the indicated time point after implantation. Mice treated with an estrogen pellet or their sham-operated control mice were killed 8 days after implantation. Testosterone powder (Steraloids, Wilton, NH) was dissolved in sesame oil (Sigma) to make a 10 mg/ml final stock solution. Sonication was used to fully dissolve the steroid. For the dose-response study, female mice were anesthetized with metofane and injected subcutaneously with different doses of liquid testosterone made from the stock solution (10 mg/ml) or with vehicle. After daily injection of testosterone liquid or vehicle for 1–5 days, the mice were killed. For the time course of testosterone induction and decay of KAP-hAGT mRNA, female KAP-hAGT transgenic mice were subcutaneously injected with liquid testosterone (50 μg·g body wt⁻¹·day⁻¹) for 1–5 days. These mice were killed at different days post-testosterone treatment.

Flutamide powder (Sigma) was dissolved in a 1:1 (vol/vol) mixture of absolute ethanol and sesame oil. For male mice, different doses of liquid flutamide or ethanol/oil vehicle were injected subcutaneously. After daily injection for 4 days, the mice were killed. For female mice, a testosterone pellet (5 mg testosterone designed for 21-day release) was first subcutaneously implanted. After 4 days of testosterone treatment, these mice were subcutaneously injected with liquid flutamide (4 g). After 4 days of daily injection, the mice were killed.

Male mice were anesthetized with metofane and cleaned at the scrotum with ethanol. A 1-cm median incision was made at the tip of the scrotum. The testes lying in the sacs can be seen by placing pressure on the lower abdomen. A 5-mm incision was made into each sac, and the testis, epididymis, vas deferens, and spermat blood vessels were pulled out. A single ligature was placed around the spermatic blood vessels and vas deferens. The testis and epididymis were removed by severing the blood vessels and vas deferens distal to the ligature. The remaining vas deferens was pushed back into the scrotum, and the incision was sutured. The castrated mice were put on a heating pad to recover. The whole surgery lasted <10 min. Mice were killed 1–7 days after castration. Some castrated mice were subcutaneously implanted with a testosterone pellet (5 mg testosterone designed for 21-day release) 8 days after castration and were killed 4 days later.

**Analysis of gene expression.** Northern blot analysis was used to examine the expression of hAGT and KAP in kidneys of KAP-hAGT transgenic mice. Kidney samples were removed from mice, frozen on dry ice, and stored in −80°C. Total kidney RNA was isolated as previously described (9). Total kidney RNA (20 μg) was separated by 1.5% agarose gel and transferred to a support nitrocellulose membrane. RNA blots were hybridized with a 32P-labeled antisense hAGT RNA probe transcribed from a partial cDNA that was derived from exon 2 of the hAGT gene at nucleotides 302–819 relative to the transcription start site or a 32P-labeled antisense KAP RNA probe transcribed from a partial cDNA clone encompassing coordinates 93–521. Most blots were performed in duplicate.

An RNase protection assay (RPA) was performed to quantify the expression of hAGT in the kidney of KAP-hAGT transgenic mice. A Hyb-Speed RPA kit (Ambion) was used according to the manufacturer’s protocol using 20 μg total kidney RNA. The full-length probes for hAGT and mouse β-actin genes are nucleotides 630 and 330, respectively, and the expected protected fragments are nucleotides 518 and 250, respectively. The RPA bands were quantified by using a Molecular Dynamics Storm 820 PhosphorImager system and the ImageQuant Version 4.0 software provided by the manufacturer. hAGT mRNA expression was normalized relative to mouse β-actin in each RPA reaction.

For immunohistochemistry analysis, mice were perfused with PBS buffer in the circulation immediately after they were killed by CO₂ asphyxiation. The fixing solution (4% paraformaldehyde, 0.5% glutaraldehyde, 100 mM sodium phosphate buffer, pH 7.4) was then perfused to replace PBS and to fix tissues. Kidneys were isolated, fixed in the same solution for 2 h, and immersed in 30% sucrose solution overnight at 4°C. Kidneys were frozen in OCT on dry ice and sectioned at 8–10 μm. Slides were first rinsed with Superblock (Pierce) for 5 min and incubated with 0.1% Triton X-100 in Superblock for 10 min at room temperature. After permeabilization by Triton X-100, slides were then incubated with rabbit anti-hAGT primary antibody diluted 1:1,000 in 0.1% Triton X-100 in Superblock overnight at 4°C. Slides were then washed with PBS for 10 min and incubated with
secondary indocarbocyanine-labeled donkey anti-rabbit antibody diluted 1:500 in PBS at 37°C for 2 h. Slides were washed again with PBS and mounted with a coverslip. Confocal microscopy was performed with a Bio-Rad MRC-1024 Hercules laser scanning confocal microscope equipped with a Kr/Ar laser.

RESULTS

Our previous results show that the KAP-hAGT transgene is constitutively expressed in the kidney of adult male mice. To further study transgene expression in males, we examined the time course of KAP-hAGT expression after birth and during the first few weeks of life (Fig. 1). Human AGT expression was very low from newborn to 3 wk of age but dramatically increased at 4 wk of age, reaching a maximum between 6 and 8 wk of age. This temporal pattern of expression of KAP-hAGT is similar to that of the endogenous KAP gene and parallels serum testosterone levels in rodents, suggesting strong androgen regulation (1, 20).

We performed a dose-response to testosterone in female KAP-hAGT mice to determine the range of transgene induction possible by exogenous testosterone. Transgene expression was gradually increased by daily injection (3 days) of testosterone within the concentration ranging from 5 to 100 μg/g body wt (Fig. 2A). An increase was also observed when the dose of testosterone was held constant (50 μg/g body wt) and different times of injection were examined (Fig. 2B). Human AGT expression reached a maximum after 5 days. Endogenous KAP mRNA also increased in a dose- and time-dependent manner although the level of induction was less than the transgene. In addition to testosterone, the endogenous KAP gene is estrogen responsive (23). High doses of estrogen (1.2–9.5 mg/day) induced the expression of the transgene and endogenous KAP gene in female KAP-hAGT mice (Fig. 3). We next considered that the most useful application for a regulated promoter such as KAP would be if transgene expression could be repressed as well as induced. We therefore examined the time course of transgene mRNA decay once androgen administration was discontinued. One group of female mice was treated for 1–5 days with 50 μg/g body wt−1⋅day−1 testosterone as above, and a second group was treated for 5 days (daily injection) and then allowed to recover from testosterone treatment for 1–8 days. As above, KAP-hAGT mRNA was induced to a maximal level after 5 days of treatment (Fig. 4A). Expression of transgene mRNA decreased steadily after testosterone was discontinued (by 32, 65, 85, and 90%, respectively, for days 1–4) and could not be detected 5 days after testosterone treatment was halted. A graphic representation of the data is shown in Fig. 4B. In contrast, the endogenous KAP mRNA retained its baseline expression level after testosterone treatment was halted. These data indicate that the KAP promoter employed in this transgene is 1) androgen-responsive, 2) much more dependent on androgen than the endogenous
KAP promoter, and 3) can be turned both “on” and “off” by manipulating androgen levels.

To determine whether transgene expression could be similarly manipulated in males, we examined its response to castration and readdition of testosterone, and its response to androgen receptor antagonism. Transgene mRNA gradually diminished with castration and was below the level of detection 4 days after castration. Testosterone administration to castrated male mice caused a superinduction of transgene expression above the baseline observed in normal males. KAP expression also decreased with castration but remained at easily detectable levels throughout the experiment. Testosterone administration to castrated male mice caused a superinduction of transgene expression above the baseline observed in normal males (Fig. 5A). Daily injection of the androgen-receptor antagonist flutamide for 4 days caused a dose-dependent decrease in transgene mRNA (Fig. 6A). In fact, flutamide treatment (2 g/day) reduced transgene mRNA levels to undetectable levels. Moreover, administration of flutamide (4 g/day) blunted the increase in transgene expression caused by testosterone treatment of female mice (Fig. 6B).

Finally, cell-specific expression of hAGT protein was examined by immunohistochemistry (Fig. 7). Fluorescent signals specific to hAGT protein were restricted to the proximal tubule cells in KAP-hAGT males. No signals were observed in controls, suggesting that the antibody was specific to hAGT. As expected, there was no positive staining in KAP-hAGT females. Importantly, hAGT protein was localized to the proximal tubule cells in KAP-hAGT females treated with estrogen (9.5 mg/day, F+T) or testosterone (0.24 mg/day, F+T). Consistent with our Northern assay and RPAs, a stronger signal was observed in KAP-hAGT females treated with testosterone than estrogen. The results indicate that hormonal treatment does not alter the cell-specificity of KAP-hAGT expression, thus validating the use of estrogen, androgen, and androgen-receptor antagonists as “molecular switches” to regulate the expression of this and other KAP promoter-controlled transgenes.

DISCUSSION

The major finding of the present study is that the expression of the KAP-hAGT transgene is androgen dependent and is markedly responsive to both increases or decreases in androgen. In addition, high doses of estrogen can also stimulate KAP-hAGT expression in female mice. These data suggest that the level of KAP promoter activity in transgenic mice can be controlled both temporally and in magnitude by manipulating the levels of androgen. Consequently, the fortuitous androgen regulation of this promoter can be used as a molecular “on-off” switch to control transgene expression in this model.

The KAP promoter contains elements with homologies to consensus sequences for regulatory motifs such as an ERE, ARE, and TRE; and the gene is stimulated by androgen, estrogen, and thyroid hormone (23). The KAP-hAGT transgene used in our study contains 1,542 bp of the KAP promoter fused to a 10.3-kb hAGT genomic clone encompassing exons II-V and including 1) a 70-bp segment derived from the 3’ end of intron I, 2) introns II-IV, and 3) the native 1.4-kb 3’ flanking sequence containing the poly(A) sites. The promoter contains an ARE at position −39, an ERE at −1189,
two half-palindromic ERE sites at -604 and -264, and a TRE at -609 (28). In the present study, we indicate that the KAP-hAGT transgene, like the endogenous KAP gene, is responsive to androgen. However, although qualitatively similar, the induction of transgene expression by testosterone in KAP-hAGT mice is quantitatively much greater than that of the endogenous KAP gene. After androgen treatment, hAGT expression was increased by 40-fold, but KAP expression was only increased 4-fold. Moreover, the baseline level of transgene expression in females is much lower than baseline KAP mRNA, and the decrease in transgene mRNA caused by castration is much greater than KAP mRNA. Therefore, whereas the KAP gene is androgen responsive, the transgene is androgen dependent.

One potential explanation for high-level induction of the transgene in response to androgen is that the 10.3-kb hAGT genomic clone used in our construct contains AREs or androgen sequences involved in androgen-mediated increases in transgene mRNA stability. Indeed, the hAGT gene itself is strongly androgen responsive in kidney (37). Thus the high magnitude of induction caused by testosterone might be due to combined effects from AREs or other similar sequences present in both the KAP promoter and within the hAGT gene. Interestingly, the identification of two enhancer elements in the vicinity of hAGT exon V has been reported (26, 27). Alternatively, the lower baseline levels of transgene mRNA may involve thyroid hormones, as several studies suggest that thyroid hormones are necessary to maintain basal KAP expression. In thyroid hormone-deficient hty/hyt mice, KAP gene expression follows a pattern similar to that observed for the KAP-hAGT transgene (32, 33). In untreated hty/hyt females, KAP expression is undetectable but is restored to normal levels by thyroid hormone administration. Male hty/hyt mice have normal baseline KAP expression, which is diminished to

Fig. 6. Transgene expression in response to flutamide. Expression of the transgene (KAP-hAGT), endogenous KAP, and 18S rRNA was examined in kidney from adult male (M) and female (F) mice. A: male mice were treated with either daily vehicle (V) or flutamide at the indicated dose (g) for 4 days. B: female mice were treated with either vehicle (V), testosterone (T; 5-mg pellet designed for 21-day release) for 4 days or were first treated with testosterone for 4 days followed by daily injection of flutamide (4 g) daily for 4 days (4+T).

Fig. 7. Cell-specific expression of the transgene. Confocal immunofluorescence images of kidney from nontransgenic (C), untreated female (F), estrogen-treated female (F+E), testosterone-treated female (F+T), and male (M) transgenic mice are shown. Top row: low-power micrographs (×4). Bottom row: high-power (×20) micrographs. Staining is in bright orange.
undetectable levels by castration. The 1,542-bp KAP promoter used in the present study contains a putative TRE at −609 and two TGACC motifs at −604 and −264, which have been described as sequences able to bind thyroid hormone receptors in other genes (30). Nevertheless, it is not clear whether these TRE sequences are sufficient for thyroid hormone to exert its complete response. It remains possible that elements responsive to thyroid hormone exist further 5’ of −1542 or within the KAP gene itself.

Increasing estrogen levels above normal caused an increase in endogenous KAP expression but only at high doses (23). Like endogenous KAP expression, there was no induction of transgene expression in KAP-hAGT females administered estrogen at the doses lower than 0.48 mg/day. Only at high doses (1.2–9.5 mg/day) was the induction of hAGT expression observed, suggesting the KAP promoter may not respond to physiological doses of estrogen but can be stimulated by high doses of estrogen. Indeed, ovariectomy of mice does not eliminate KAP gene expression and, in fact, results in a slight increase (23), further suggesting estrogen in physiological doses may have no effect on the induction of the KAP promoter. Because estrogen has only a modest stimulatory action on hAGT, it is clear that it will not be a useful tool to activate the transgene.

The strong androgen dependence of the KAP-hAGT transgene motivated our long-term goal of developing an inducible hypertension model in which blood pressure levels could be manipulated by exogenous hormone. The role of the renin-angiotensin system in the regulation of blood pressure and sodium and water homeostasis is well recognized. AGT is the only known substrate for the enzyme renin. Because the level of AGT in humans is close to the Michaelis-Menten constant value $K_m$ for renin (16), AGT levels can dictate the activity of the RAS, and its upregulation may cause an increase in blood pressure. There is compelling evidence that perturbations in AGT synthesis result in changes in RAS activity and blood pressure. First, one haplotype of AGT shows a strong correlation with plasma AGT and hypertension (15, 16). Next, injection of antisense AGT oligodeoxynucleotides decreases plasma AGT, ANG-II, and blood pressure in the spontaneously hypertensive rat (14). Transgenic mice containing both hREN and hAGT exhibit high plasma AGT, ANG-II, and blood pressure in the spontaneously hypertensive rat (14). Transgenic mice containing both hREN and hAGT exhibit high plasma AGT, ANG-II, and chronic hypertension (21). Finally, plasma AGT levels and blood pressure were found to increase progressively in mice containing zero to four copies of the AGT locus (31). Thus we can use these mice as models of inducible hypertension by manipulating hAGT expression in them. We reported that male transgenic mice containing both KAP-hAGT and hREN exhibit chronic hypertension, and blood pressure in females can be increased in response to androgen treatment (7). Testosterone caused an increase in blood pressure in female double transgenic mice, 19 mmHg after 1 day, 31 mmHg after 2 days, and peaking at 40 mmHg (149 ± 4 mmHg) after 3 days. Testosterone did not cause any change in blood pressure in the control group. Thus altering androgen levels in hREN/ KAP-hAGT double transgenic mice provides temporal control over the onset and duration of hypertension. Such an inducible model would provide a major advantage over presently existing transgenic models of hypertension, in which the transgenes are activated at birth and are expressed throughout life, and provide an opportunity to examine the physiological consequences of hypertension on organ function and end-organ damage in mice with varied exposure to hypertension.

We acknowledge the outstanding technical assistance of Kelly Andringa, Patricia Lovell, Lucy Robbins, and Norma Sinclair for generation and genotyping of KAP-hAGT transgenic mice. Deborah Davis for assistance with timed breedings, and Henry Keen and Robin Davison for reviewing the PhD dissertation resulting in this manuscript.

Funds in support of this work were obtained from the National Heart, Lung, and Blood Institute (HL-55006 and HL-48058). C. D. Sigmund was an Established Investigator of the American Heart Association. Y. Ding was a predoctoral fellow of the American Heart Association-Heartland Iowa (Heartland) Affiliate. Transgenic mice were generated and maintained at the University of Iowa Transgenic Animal Facility, which is supported, in part, by the College of Medicine and the Diabetes and Endocrinology Research Center. DNA sequencing was performed at the University of Iowa DNA Core Facility.

REFERENCES


