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Dexamethasone Promotes Hypertension by Allele-specific Regulation of the Human Angiotensinogen Gene*[S]

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Varunkumar G. Pandey[‡§], Sudhir Jain[‡], Anita Rana[‡], Nitin Puri[‡], Sri Krishna C. Arudra[¶], Brahmaraju Mopidevi[‡], Meenakshi Kaw[‡], Alberto Nasjletti[‡], and Ashok Kumar[‡1]

From the Departments of [‡]Physiology and Pharmacology and [¶]Pathology, College of Medicine, University of Toledo, Toledo, Ohio 43614 and the [§]Department of Pharmacology, New York Medical College, Valhalla, New York, 10595

Background: Glucocorticoids modulate the RAS and cause hypertension.
Results: SNPs in the hAGT promoter form two haplotypes, −6A and −6G. Transgenic mice with haplotype −6A respond to dexamethasone with tissue-specific up-regulation of hAGT, increased plasma AngII, and hypertension.
Conclusion: Haplotypes of the hAGT gene govern transcriptional response to dexamethasone.
Significance: Polymorphisms in hAGT provide for genetic predisposition to glucocorticoid-induced hypertension.

The human angiotensinogen (hAGT) gene has polymorphisms in its 2.5-kb promoter that form two haplotype (Hap) blocks: −6A/G (−1670A/G, −1562C/T, and −1561T/C) and −217A/G (−532T/C, −793A/G, −1074T/C, and −1178G/A). Hap −6A/−217A is associated with human hypertension, whereas Hap −6G/−217G reduces cardiovascular risk. Hap −6A/−217A has increased promoter activity with enhanced transcription factor binding, including to the glucocorticoid receptor (GR). Glucocorticoid therapy frequently causes hypertension, the mechanisms for which are incompletely understood. We have engineered double transgenic (TG) mice containing the human renin gene with either Hap of the hAGT gene and examined the physiological significance of glucocorticoid-mediated allele-specific regulation of the hAGT gene. We have also studied the consequential effects on the renin angiotensin system and blood pressure. TG mice with Hap −6A and −6G were treated with and without a low dose of a GR agonist, dexamethasone (2.5 μg/ml), for 72 h. We found greater chromatin-GR binding with increased GR agonist-induced hAGT expression in liver and renal tissues of Hap −6A mice. Additionally, dexamethasone treatment increased circulating hAGT and angiotensin II levels in Hap −6A mice, as compared with −6G mice. Importantly, GR agonist significantly increased blood pressure and redox markers in TG mice with Hap-6A of the hAGT gene. Taken together, our results show, for the first time, that glucocorticoids affect hAGT expression in a haplotype-dependent fashion with SNPs in Hap −6A favoring agonist-induced GR binding. This leads to increased expression of the hAGT, up-regulation of the renin angiotensin system, and increased blood pressure and oxidative stress in Hap −6A mice.

Hypertension is a polygenic disorder with up to 60% heritability (1, 2). Complex environmental-genetic cross-talk underlies this inheritance and affects phenotype in the majority of patients. Single-nucleotide polymorphisms (SNPs) in the genome provide interindividual variability in these gene-regulatory networks, wherein transcriptional regulation of the key genes is altered under pathophysiological settings. In this regard, we have recently established SNP-directed variations in human AGT[2] (hAGT) gene expression. AGT is sequentially cleaved by renin and angiotensin-converting enzyme to form the octapeptide, angiotensin II (AngII). AngII stimulates aldosterone release from the adrenal cortex, and together this constitutes the renin-angiotensin-aldosterone axis. Renin-angiotensin-aldosterone axis overactivity leads to volume overload, hypertension, and remodeling of the cardiovascular and renal systems.

The hAGT gene has a group of polymorphisms in its 2.5-kb promoter that form two sub-Hap blocks. The first subblock contains SNPs at −6, −1670, −1562, and −1561 sites that are in linkage disequilibrium (LD). Variants −1670A, −1562C, and −1561T almost always occur with −6A, whereas the variants −1670G, −1562G, and −1561G almost always occur with −6G (3). The second subblock contains SNPs at −217, −532, −793, −1074, and −1178. Variants −532T, −793A, −1074T, and −1178G are in LD with −217A, whereas variants −532C, −793G, −1074C, and −1178A sites are in LD with −217G. Taken together, there are four possible Haps for the hAGT gene: −6A/−217A, −6G/−217A, −6A/−217G, and −6G/−217G (3–7). The highest risk for human hypertension is conferred by the −6A/−217A Hap, whereas lowest risk is conferred by Hap −6G/−217G (7). We have engineered transgenic (TG) mice with −6A/−217A and −6G/−217G Haps to study the transcriptional regulation and associated cardiovascular implications of these variants of the hAGT gene, termed Hap −6A and −6G, respectively (3). TG mice with hAGT Hap −6A have significantly higher baseline blood pressure as compared with TG mice with hAGT Hap −6G. This increase in baseline

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[1] To whom correspondence should be addressed: Rm. 351, 3000 Arlington Ave., MS 1008, Dept. of Physiology and Pharmacology, University of Toledo Health Science Campus, Toledo, OH 43614. Tel.: 419-383-4520; Fax: 419-383-2871; E-mail: ashok.kumar2@utoledo.edu.

[2] The abbreviations used are: AGT, angiotensinogen; hAGT, human AGT; Hap, haplotype; TG, transgenic; LD, linkage disequilibrium; DEX, dexamethasone; AngII, angiotensin II; RAS, renin angiotensin system; GR, glucocorticoid receptor; GRE, glucocorticoid response element; SBP, systolic blood pressure; C/EBP, CCAAT/enhancer-binding protein.

blood pressure is accompanied by significantly higher hAGT expression in Hap −6A mice, with no significant change observed in the mAGT gene. The contributions of the SNP-dependent variable transcriptional regulation of the hAGT gene are highlighted by these findings.

In this regard, glucocorticoids are well known regulators of the AGT gene, especially in the liver and adipose tissue (8, 9). Increased glucocorticoid levels cause hypertension (10), mechanisms for which could include increased expression of the AGT gene. Contextually, SNPs in the "cis" element of the genes, such as the hAGT, can promote varying affinity for the GR and thus be amenable to modulation by pathophysiological levels of glucocorticoids. Differential gene regulation by endogenous or exogenous glucocorticoids, via activation of the glucocorticoid receptors (GRs), is an area of intense current research. Importantly, we have shown that the polymorphisms in Hap −6A positively affect the binding of transcriptional regulators, such as the glucocorticoid receptors, to the promoter region of the hAGT gene (3). In this study, we tested the hypothesis that glucocorticoids via activation of GR will preferentially induce the hAGT gene in Hap −6A TG mice with, consequentially, an increase in blood pressure. We show here that dexamethasone (DEX) increases hAGT gene expression via increased GR binding to the promoter of the hAGT gene in TG mice with Hap −6A, as compared with the Hap −6G. Crucially, DEX-dependent induction of the hAGT gene is accompanied by significant blood pressure elevation in TG mice with Hap −6A, an effect reversed by angiotensin receptor blockade.

MATERIALS AND METHODS

Generation of TG Mice—All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the institutional animal care and use committee at the University of Toledo. The TG mice utilized in this study were generated in our laboratory as described previously by Jain *et al.* (3). Genotyping analysis of the tail snips, followed by sequencing, was performed to confirm the genetic lineage of these TG mice. As described previously, the TG mice with Hap −6A have variants −6A, −20A, −217A, −532T, −793A, −1074T, −1178G, −1561T, −1562C, and −1670A, whereas TG mice with −6G Hap have variants −6G, −20A, −217G, −532C, −793G, −1074G, −1178A, −1561G, −1562G, and −1670G. Because hAGT is not cleaved by the mouse renin, we generated double TG mice containing either Hap −6A or −6G of the hAGT gene and human renin gene (made available by Dr. Curt Sigmund). For experimental purposes, these TG mice were housed individually. Twelve-week-old double TG mice containing Hap −6A and −6G each were divided into two groups ($n = 6$). For both Haps, experiments were performed either in the presence or absence of DEX.

DEX Treatment—Twelve-week-old male double TG mice containing Hap −6A or −6G, along with age-matched C57/BL6 mice, were divided into two groups ($n = 6$). TG mice were caged individually. DEX, at a dosage of 2.5 μg/ml in drinking water, was administered to the TG mice for 72 h. The low dose of DEX was selected to avoid the effect of DEX on the endogenous renin-angiotensin-aldosterone axis. Complementary experiments were performed with high-dose (12.5 μg/ml) and low-dose (0.25 μg/ml) DEX, examining effects on blood pressure of TG mice. Animals were maintained in a 22 °C room with a 12-h light/dark cycles and received standard chow and DEX or vehicle in drinking water *ad libitum*. No significant difference was observed in the volume of drinking water or DEX solution consumed by TG mice with either Hap −6A or Hap −6G and non-TG C57/BL6 mice.

Quantitative RT-PCR—The liver and the kidneys were harvested at the end of DEX treatment and snap-frozen in liquid nitrogen. The extracted tissues were stored at −80°C until utilized for further experiments. RNA was isolated using RNeasy Plus minikit (Qiagen). RNA (1 μg) was reverse-transcribed into cDNA using the Revert Aid first strand cDNA synthesis kit (Fermentas), as described in the manufacturer's protocol. Following a 95 °C incubation for 10 min, 40 cycles of PCR (95 °C for 30 s, 60 °C for 30 s), were then performed using 1 μl of cDNA, 50 nM PCR primers, and 12.5 μl of SYBR Green PCR Master Mix in 25-μl reactions. Threshold cycles for three replicate reactions were determined using MxPro-Mx3005P software (version 4.10), and relative transcript abundance was calculated following normalization with mouse GAPDH. Primers for human and mouse AGT and mouse GAPDH were obtained from Super Array Bioscience Corp. (Frederick, MD) or from Integrated DNA Technologies (Coralville, IA). In general, relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample, such as untreated control. This is a widely used method to present relative gene expression by the comparative C_T method, also referred to as the $2^{-\Delta\Delta CT}$ method.

Immunoblot Analysis—Protein extracts were prepared from the liver, kidneys, and plasma collected from the TG and C57/BL6 mice at the end of the experiment. Protein extracts (25 μg) were separated by SDS-PAGE (12% polyacrylamide) and transferred to 0.45-μm PVDF membranes (Millipore, Billerica, MA) for 1 h. The membranes were incubated with either hAGT (1:2000) (catalog no. 3249−1, Epitomics, Burlingame, CA) or mAGT (catalog no. 28101, IBL, Takasaki, Japan) primary antibody. Blots were developed using an infrared imaging system (Odyssey, LI-COR Biosciences, Lincoln, NE). The results for the liver and the kidneys were normalized to mouse β-actin (A2228, Sigma) and those for plasma were normalized to mouse albumin (1:10,000) (NB600-41532, Novus Biologicals).

In Vivo Chromatin Immunoprecipitation (ChiP) Analysis—The ChIP assay was performed using the EZ-ChIP assay kit from EMD Millipore. Mice were perfused with normal saline, and the liver and the kidneys were removed, washed, and fixed with 1% formaldehyde for 20 min at room temperature. The DNA was fragmented by sonication, and 10 μl of the chromatin solution was saved as input. 5 μg of anti-glucocorticoid receptor (anti-GR) or rabbit immunoglobulin G were added to the tubes containing 900 μl of sonicated chromatin solution; the mixture was incubated overnight at 4 °C. The antibody complexes were captured with the protein A-agarose beads and subjected to serial washes (as described in the manufacturer's protocol). The chromatin fraction was extracted with SDS buffer and reverse cross-linked at 65 °C for 4–6 h. The DNA was then purified as described in the manufacturer's protocol.

The immunoprecipitated DNA (1 μl) and the input DNA (1 μl) were subjected to 35 cycles (of PCR amplification (denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s) using (*a*) −314AGTfor (CTCAGGCTGTCACACAC-CTA) as a forward and −6AGTrev (TCTTCCCCTGGC-CGGGTCACGAT) as a reverse primer, when GR antibody was used to examine the binding of GR at the −217 position of the hAGT gene, or (*b*) −1757AGTfor (CAGGCACAGTGG-AAACTCTCC) as a forward primer and −1554AGTrev (AGTAACAAGTCCACCTGGAC) as a reverse primer, when GR antibody was used to examine the binding of GR across the −1561 and −1562 polymorphic sites of the hAGT gene. The PCR-amplified products were analyzed on 2% agarose gel. The relative band intensities by densitometry were measured using Quantity One quantitation software from Bio-Rad, and the results were normalized to the band intensities from the respective input DNA. The fraction enriched by rabbit IgG was used as a negative control for nonspecific binding. A primer set that amplified the DNA fragment of the hAGT gene with no known glucocorticoid response element was used as a nonspecific control for GR-enriched DNA fragments.

Measurement of Plasma Angiotensin II—Plasma angiotensin-II levels were determined by an ELISA kit purchased from Ray Biotech, Inc. The angiotensin II concentration in the samples was determined directly from the standard curve according to the manufacturer's protocol as described previously (3).

Blood Pressure Measurement in TG Mice—Blood pressure was measured in the conscious state by radio telemetry as described previously (3). Systolic blood pressure was continuously acquired by implantation of telemetric probe PA-C20 into the aorta via the left carotid artery. After 1 week of recovery from the surgical procedure, blood pressure readings were recorded every 10 min using Data-Dataquest ART software purchased from Data Science International. Baseline blood pressure was collected for 7 days, followed by blood pressure measurements in response to DEX treatment, with and without losartan.

Statistical Analyses—All experiments were conducted with six animals in each group. Data are expressed as the means ± S.E. Statistical significance was assessed using two-way analysis of variance with a Tukey-Kramer post hoc analysis. The significance level was set at ($p < 0.05$).

RESULTS

DEX Treatment Promotes Greater GR Binding to the Chromatin of −6A Hap Mice—The hAGT gene polymorphisms at −217 and −1561 in −6A HapTG mice have stronger homology with consensus GRE as compared with Hap −6G (Fig. 1); therefore, we examined the effect of DEX on the binding of GR to the −217 and −1561/−1562 region of the hAGT gene in both TG animals. A ChIP assay was performed using GR-specific antibody on chromatin extracts from hepatic and renal tissues of the TG animals. As shown in Fig. 2, GR binds more strongly to the chromatin obtained from hepatic tissue of TG mice with Hap −6A, as compared with Hap −6G. This effect is further increased by DEX treatment, where GR enrichment at these sites increases further ($p < 0.05$) only in TG mice containing Hap −6A. Similar results were observed in chromatin isolated from renal tissues of these TG animals (Fig. 3). These results

FIGURE 1. **Schematic showing the homology in the promoter of hAGT gene containing either Hap −6A or Hap −6G with the GR consensus binding site (GRE) at the −217 position (*A*) and −1561/−1562 position (*B*).** * marks the SNP site.

show increased GR binding to the hAGT promoter in the liver and kidney of TG mice containing Hap −6A, an effect accentuated by treatment with a GR agonist. The effect of DEX on the chromatin of TG mice containing Hap −6G was not statistically significant. Our previous transfection assays have shown that DEX treatment slightly increased the promoter activity of reporter construct containing −6G Hap. However, this small increase in the promoter activity of −6G Hap may be attributed to co-transfection of GR in these experiments.

DEX Treatment Increases hAGT Expression in Liver and Kidneys of −6A Hap Mice—Transcriptional effects of the increased GR-GRE interaction were examined by gene expression analysis. Liver is the major source of circulating AGT, and renal production of AGT governs the tissue RAS. Together, plasma AGT and tissue RAS critically regulate the extracellular volume and long term set point of the mean arterial pressure. Liver (Fig. 4, *A* and *C*) and renal (Fig. 4, *B* and *D*) extracts both show increased ($p < 0.05$) baseline hAGT mRNA levels in the Hap −6A. Importantly, GR agonist (DEX) further induced hAGT transcription ($p < 0.05$) in the −6A Hap without any significant induction in the −6G Hap (results from individual experiments for mRNA expression of hAGT in the liver and the kidneys are provided as dot plots in supplemental Fig. S1). This observation corroborates the results of the ChIP analyses showing higher GR-GRE interaction in the hAGT of the −6A Hap. Furthermore, the specificity of GR induction of the hAGT gene is reflected in experiments showing no transcriptional effect of DEX on the mAGT mRNA level under these conditions.

Complementary immunoblot analysis confirmed the induction of the hAGT gene in TG mice with Hap −6A in response to treatment with DEX ($p < 0.05$) (Fig. 5). Liver (Fig. 5, *A* and *B*) and renal (Fig. 5, *C* and *D*) homogenates probed with hAGT-specific antibody show transcriptional induction of the hAGT in response to DEX treatment, an observation only evident in the −6A TG mice (results from individual experiments for the densitometric analysis are represented in dot plots for the hAGT in the liver and the kidney; supplemental Fig. S2).

DEX Treatment Increases Plasma hAGT and AngII in −6A Hap Mice—In order to discern the systemic effects of increased tissue AGT, caused by DEX treatment, we examined hAGT levels in plasma of our TG mice. As seen in Fig. 6, *A* and *B*, baseline plasma hAGT is significantly ($p < 0.05$) increased in TG mice containing Hap −6A of the hAGT gene. DEX treat-

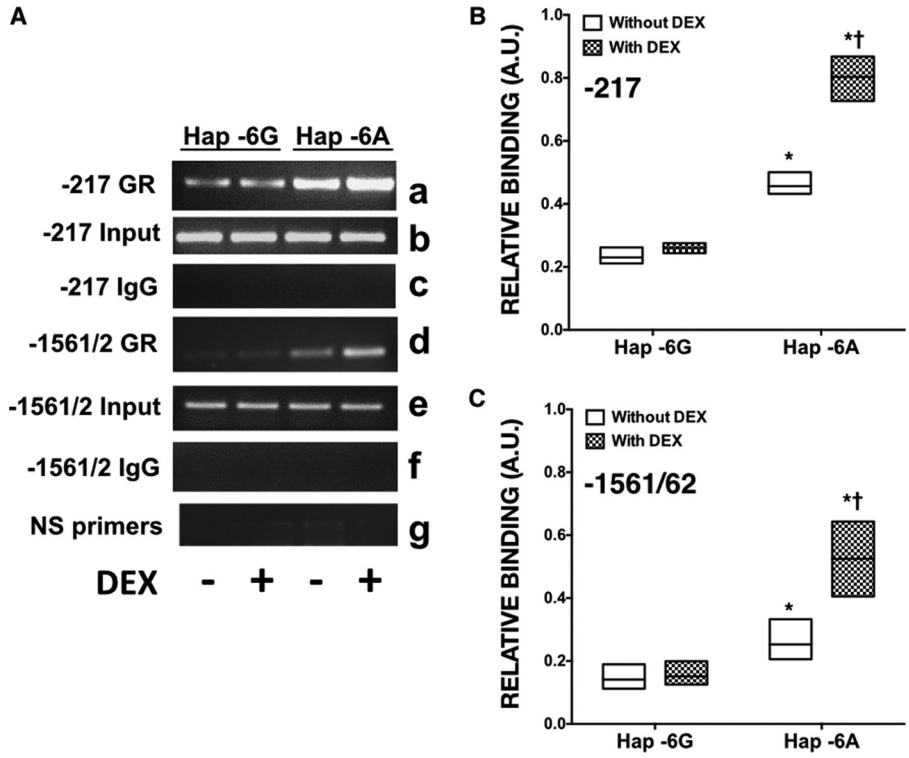

FIGURE 2. *A*, representative ChIP assay using immunoprecipitated DNA from the liver of TG mice containing either Hap −6A or −6G in the presence or absence of DEX treatment. The assay was performed in the presence of antibodies against GR (*a* and *d*), input DNA (*b* and *e*), and IgG (*c* and *f*). Immunoprecipitated DNA was used to the amplify nucleotide sequence encompassing the −217 (*a–c*) and −1561/−1562 (*d–f*) regions, respectively, as described under "Materials and Methods." *g*, a PCR using primers for a nonspecific (*NS*) region in the hAGT gene that does not have a putative GRE. Quantitation of the PCR product of GR-enriched DNA, relative to input, at the −217 (*B*) and −1561/−1562 (*C*) sites of the hAGT gene shows a significant increase in the DEX-induced GR binding in TG mice with Hap −6A. *, $p < 0.05$ compared with Hap −6G; †, $p < 0.05$ compared with Hap −6A. Results are shown as mean ± S.E. ($n = 5$). *, $p < 0.05$ *versus* untreated Hap −6G; †, $p < 0.05$ *versus* untreated Hap −6A. *A.U.*, arbitrary units.

ment significantly ($p < 0.05$) increased plasma hAGT levels in TG mice with Hap −6A while having no effect on the hAGT levels in plasma of TG mice with Hap −6G. Importantly, this low-dose DEX treatment had no significant effect on the endogenous mAGT gene in either Hap −6A (densitometric analyses with normalization to albumin: without DEX = 0.22 ± 0.01 *versus* with DEX = 0.30 ± 0.06) or Hap −6G (without DEX = 0.20 ± 0.02 *versus* with DEX = 0.22 ± 0.03) or in the C57/BL6 mice (without DEX = 0.33 ± 0.03 *versus* with DEX = 0.42 ± 0.02).

Classically, renin secretion by the juxtaglomerular apparatus has been viewed as the rate-limiting step in conversion of AGT to ANGII. However, recent studies confirm a causal link between increased AGT and increased ANGII levels, even in the absence of renin modifications. To this end, we analyzed plasma ANGII levels in our TG mice and found them to be elevated ($p < 0.05$) in TG mice with Hap −6A (Fig. 6*C*). Moreover, DEX treatment brought about further increase in ANGII levels in −6A animals only, results corroborating our hypothesis of DEX-induced modulation of the hAGT levels in this Hap (results from individual experiments for AngII levels are provided as a dot plot in supplemental Fig. S3). AngII levels were significantly ($p < 0.05$) lower in C57/BL6 mice (145 ± 27 pg/ml) and were not amenable to modulation by our low-dose DEX regimen (with DEX: 179 ± 32 pg/ml).

Effects of DEX Treatment on Physiological Parameters, Including Blood Pressure and Tissue Inflammation, in the Two TG Lines—Pathophysiological implications of DEX-induced elevated AGT, with consequential effects on ANGII, were evaluated at systemic and local levels. RAS increased mean arterial pressure, in the short term by vasoconstriction and in the long term by renal mechanisms; ANGII, also, activated the oxidative cascade, and both effects were AT1 receptor-mediated. We observed that TG mice with Hap −6A had significantly increased systolic blood pressure (SBP) as compared with Hap −6G at resting conditions. We next analyzed the effect of DEX on the SBP of both of our TG mice with Hap −6A and −6G. Fig. 7*B* shows that the elevation in SBP (Δ) after treatment with DEX (2.5 μg/ml) in TG mice with Hap −6A was significantly higher as compared with Hap −6G after 24, 48, and 72 h of data collection (results from individual experiments are shown on a dot plot; supplemental Fig. S4*A*). Importantly, the DEX-induced increase in SBP did not disrupt the regular diurnal variation (Fig. 7*A*). Each point in Fig. 7*A* represents the hourly mean from six male animals taken over a period of 24 h. The hourly mean was taken from the average of six SBP data points at an interval of 10 min each. It is important to note that although DEX increased blood pressure in C57/BL6 mice (without DEX = 94 ± 6 mm Hg *versus* with DEX = 103 ± 4 mm Hg), the results did not achieve statistical significance.

We further increased the dosage of DEX to 12.5 μg/ml in drinking water to investigate whether the DEX-dependent differential regulation of the blood pressure is maintained at higher dosages. We found a steep rise in the SBP of TG mice containing Hap −6A, raising it to ~165 mm Hg, whereas the TG mice with Hap −6G showed a maximum of ~140 mm Hg (Fig. 7*C*). DEX-dependent changes in SBP were prevented in

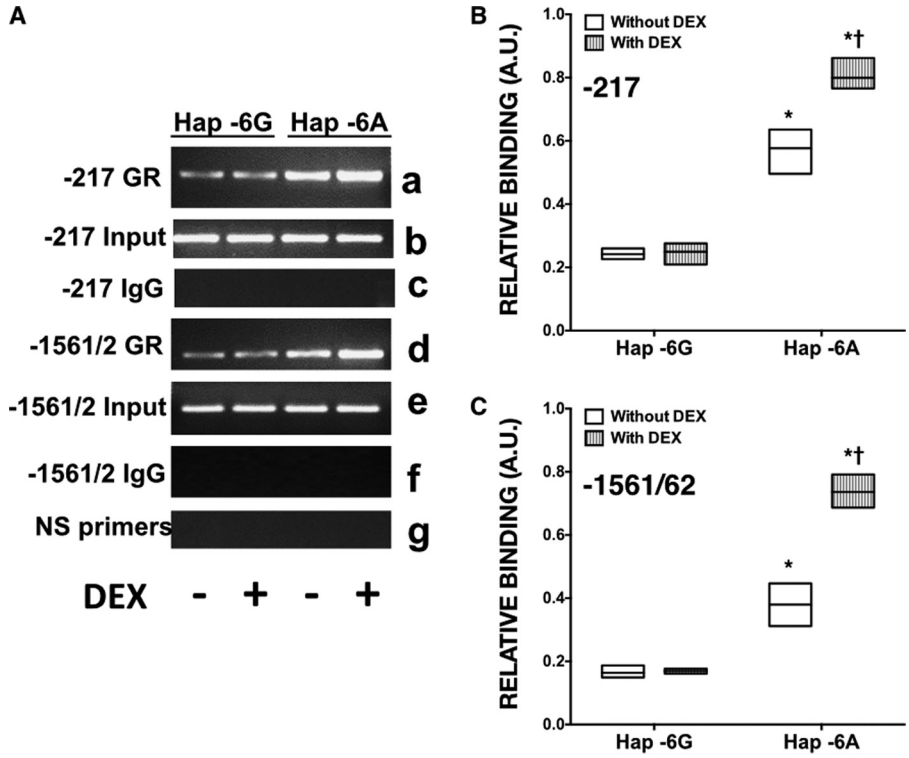

FIGURE 3. *A*, representative ChIP assay using immunoprecipitated DNA from the kidneys of TG mice containing either Hap −6A or −6G in the presence or absence of DEX treatment. The assay was performed in the presence of antibodies against GR (*a* and *d*), input (*b* and *e*), and IgG (*c* and *f*). Immunoprecipitated DNA was used to amplify nucleotide sequence encompassing the −217 (*a–c*) and −1561/−1562 (*d–f*) regions, respectively, as described under "Materials and Methods." *g*, PCR using primers for a nonspecific (*NS*) region in the hAGT gene that does not have putative GRE. Quantitation of the bands was performed as described in the legend to Fig. 2. The PCR product of GR-enriched DNA relative to input at the −217 (*B*) and −1561/−1562 (*C*) site of the hAGT gene shows a significant increase in the DEX-induced GR binding in TG mice with Hap −6A. Results are shown as mean ± S.E. ($n = 5$). *, $p < 0.05$ *versus* untreated Hap −6G; †, $p < 0.05$ *versus* untreated Hap −6A. *A.U.*, arbitrary units.

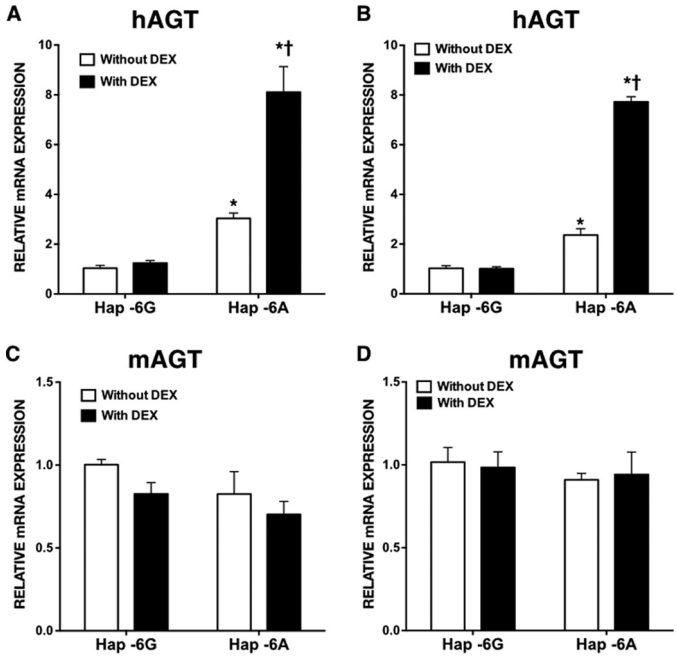

FIGURE 4. **mRNA expression of the hAGT and mAGT in liver and kidney of TG mice by quantitative RT-PCR analysis.** *A* and *C*, mRNA expression of the hAGT and the mAGT gene, respectively, in the liver of TG mice treated with and without DEX (2.5 μg/ml). *B* and *D*, mRNA expression of the hAGT and the mAGT gene, respectively, in the kidneys of TG mice treated with and without DEX (2.5 μg/ml). Results are shown as mean ± S.E. (*error bars*) ($n = 6$). *, $p < 0.05$ *versus* untreated Hap −6G; †, $p < 0.05$ *versus* untreated Hap −6A.

animals concurrently treated with an AT1 receptor blocker (losartan) (Fig. 7*D* and dot plot in supplemental Fig. S4*C*). On the other hand, low-dose DEX had no significant effect on blood pressure in any treated group (supplemental Fig. S4*B*). These experiments implicate a DEX-dependent increase in AGT and ANGII in bringing about changes in SBP in TG mice with Hap −6A. In line with historical evidence, complementary studies showed increased ($p < 0.05$) levels of oxidative markers (NOX1 (NADPH oxidase)) and attenuation ($p < 0.05$) of antioxidant defenses (SOD1 (superoxide dismutase type I)) in animals of the −6A Hap undergoing DEX treatment (Fig. 8).

CEBPβ expression is measured as a negative control to ascertain the specificity of haplotype-dependent effects of DEX on the hAGT, in our transgenic lines. As shown in Fig. 8*C*, hepatic CEBPβ expression is up-regulated by DEX treatment in all three groups, C57, Hap −6G, and −6A TG mice. Importantly, the DEX-induced increase in CEBPβ is similar in all treated groups (dot plot analysis in supplemental Fig. S5). These results corroborate that the stimulation of hAGT expression observed in response to DEX in Hap −6A mice is indeed haplotype-dependent. These results are a clear indication of the susceptibility of the −6A Hap to DEX-induced RAS activation and associated pathophysiologic alterations.

DISCUSSION

Glucocorticoid therapy is the cornerstone of treatment of diseases with chronic inflammation. DEX is widely employed in the clinics and is as frequently associated with pharmacological

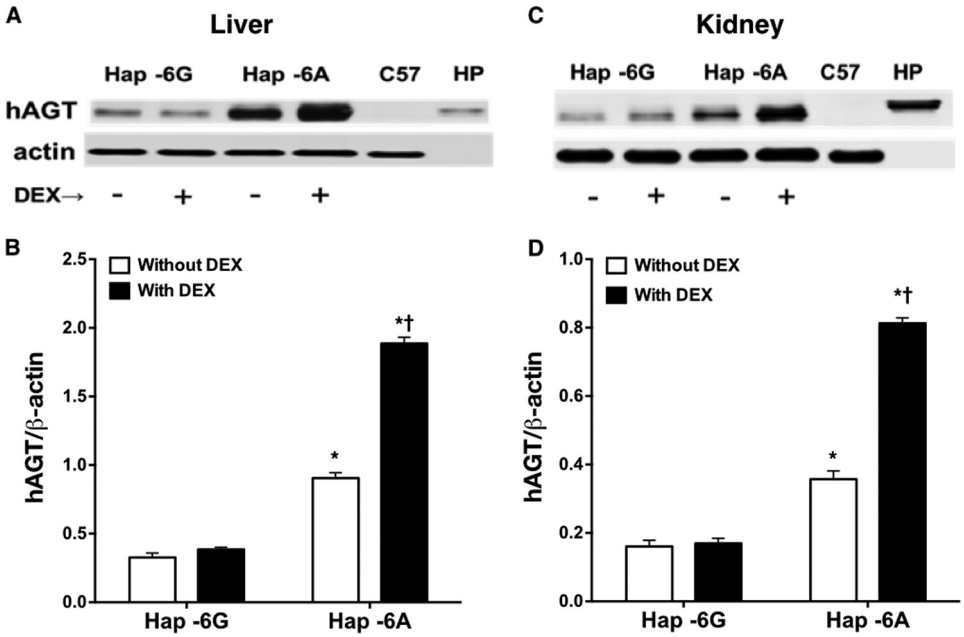

FIGURE 5. **Immunoblot analysis of the hAGT protein in liver (*A* and *B*) and kidney (*C* and *D*) of the TG mice.** The *top lanes* show representative immunoblots, whereas the densitometric analysis is depicted at the *bottom*. *Lane C57* shows liver or kidney protein extracts from c57 mice and acts as a negative control. *Lane HP* shows human plasma as a positive control for hAGT. Results are shown as mean ± S.E. (*error bars*) ($n = 5$). *, $p < 0.05$ *versus* untreated Hap −6G; †, $p < 0.05$ *versus* untreated Hap −6A.

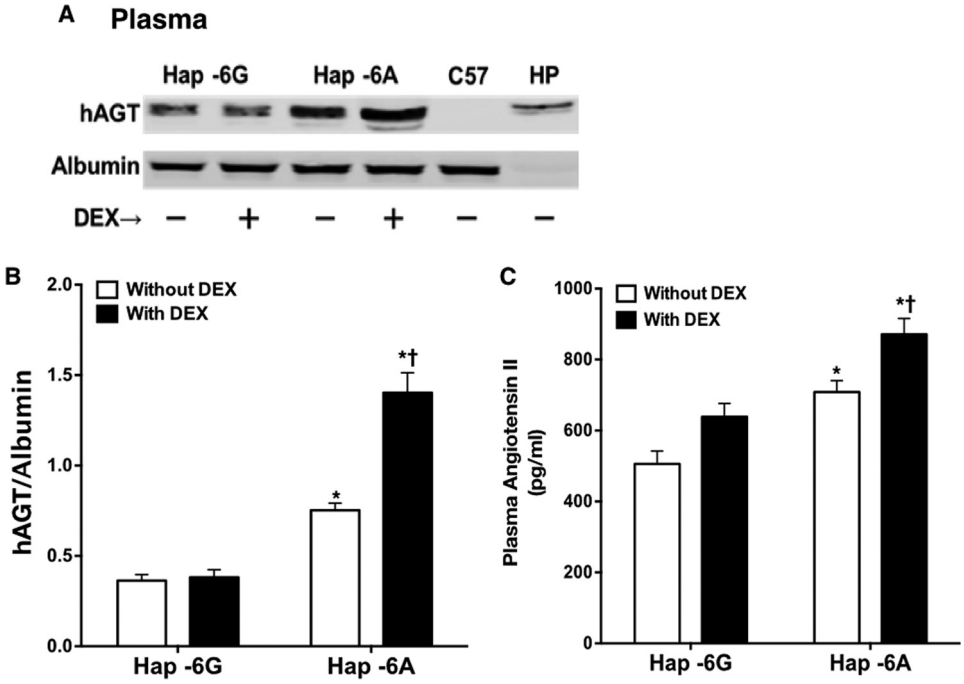

FIGURE 6. **Plasma hAGT (*A* and *B*) and AngII (*C*) level in TG mice containing either Hap −6A or Hap −6G of the hAGT gene in the presence or absence of DEX treatment.** *A*, representative immunoblot; *B* and *C*, densitometric analysis. Results are shown as mean ± S.E. (*error bars*) ($n = 5$). *, $p < 0.05$ *versus* untreated Hap −6G; †, $p < 0.05$ *versus* untreated Hap −6A.

side effects, including osteoporosis, secondary infections, diabetes, preeclampsia, and hypertension. Hypertension can be caused by RAS overactivity (11). The AGT gene is frequently linked with hypertension in humans, especially the M235T locus that is in LD with the −6 site on the hAGT promoter (12, 13). The novelty of the current study lies in the observation that low-dose GR agonist has Hap-dependent transcriptional effect on the hAGT gene with consequential activation of the RAS. This translates into increased blood pressure and RAS-associated pathological outcomes in TG mice with the −6A Hap of the hAGT gene.

The first key finding of the study confirms agonist-induced GR binding to the hAGT gene, *in vivo*, in a Hap-regulated fashion. We show here, for the first time, increased GR receptor binding to the hAGT promoter at the −217 and −1561/62 sites of the −6A Hap. These results are in line with our published *in*

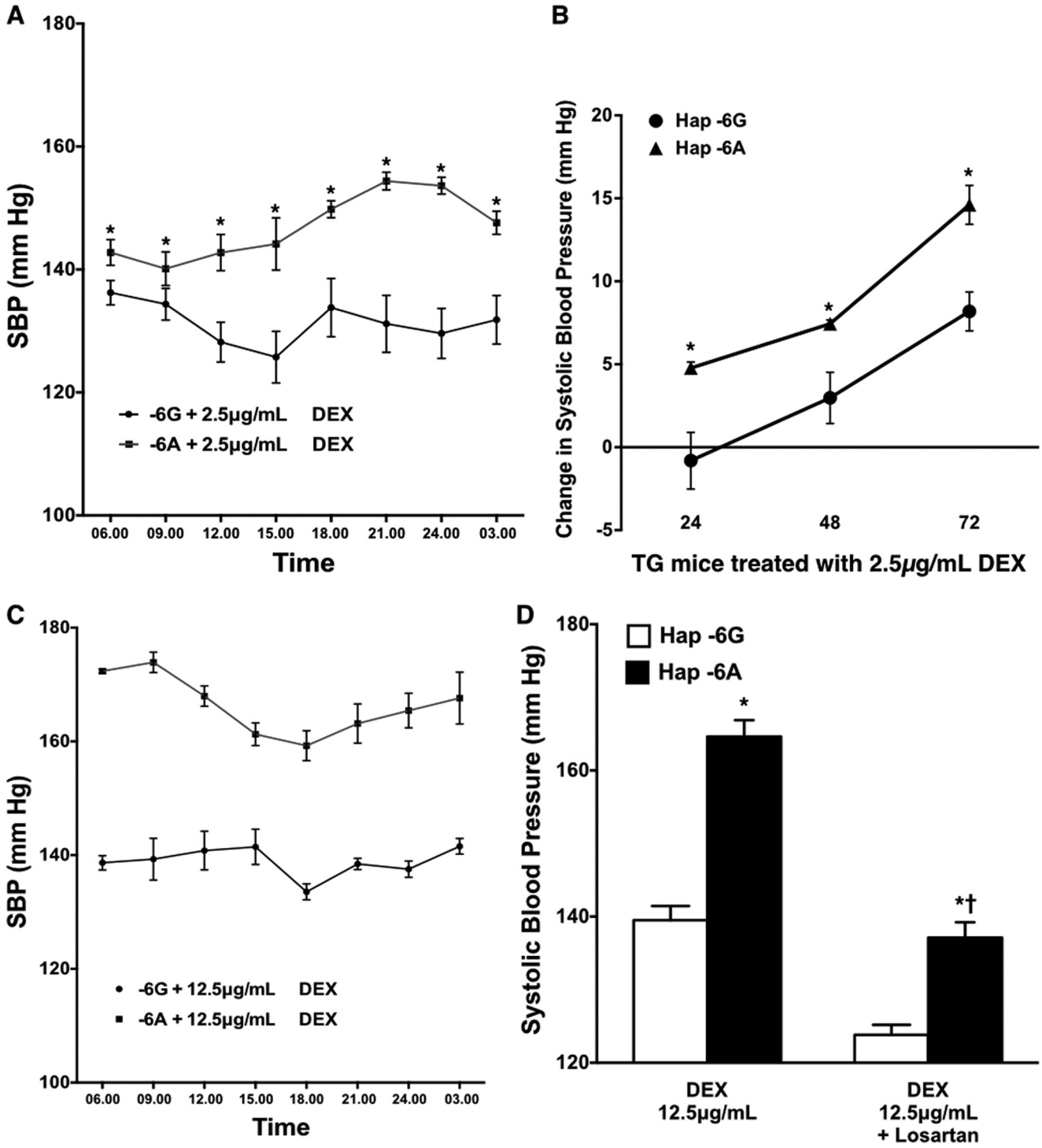

FIGURE 7. **Blood pressure analysis in TG mice.** The SBP of transgenic mice with haplotype −6A is significantly higher as compared with that of haplotype −6G after DEX treatment (*A*) over a 24-h time period. Each point represents the hourly mean from six male animals taken over 24 h. The hourly mean was taken from the average of six SBP data points at an interval of 10 min. *B*, change in systolic blood pressure over baseline after DEX treatment in Hap −6A and −6G mice; readings are averaged at 24, 48, and 72 h post-DEX (2.5 μg/ml in drinking water *ad libitum*) treatment. The difference in the elevation of SBP is further increased upon treatment with 12.5 μg/ml DEX (*C*). The DEX-induced increase in SBP was alleviated by treatment with the angiotensin type 1 receptor blocker losartan (30 mg/kg/day). Results are shown as mean ± S.E. (*error bars*) ($n = 6$). *, $p < 0.05$ *versus* Hap −6G; †, $p < 0.05$ *versus* Hap −6A in the absence of losartan.

vitro reports showing GREs in the hAGT gene that bind GR with increased affinity when the former has SNP variants associated with −6A and −217A (3, 14) (see our previous *in vitro* studies (3)). Other investigators have established both, *in vitro* and *in vivo*, glucocorticoid-mediated regulation of the AGT gene (15). Glucocorticoids have been shown to increase AGT expression in the liver and adipocytes (16–18). However, this is the first report characterizing glucocorticoid-induced, Hap-dependent regulation of the hAGT gene, especially in the kidneys. The SNPs at the −217, −1561, and −1562 sites in the Hap −6A provide greater homology for the GRE consensus sequence and promote stronger GR binding. The ChIP assay clearly demon-

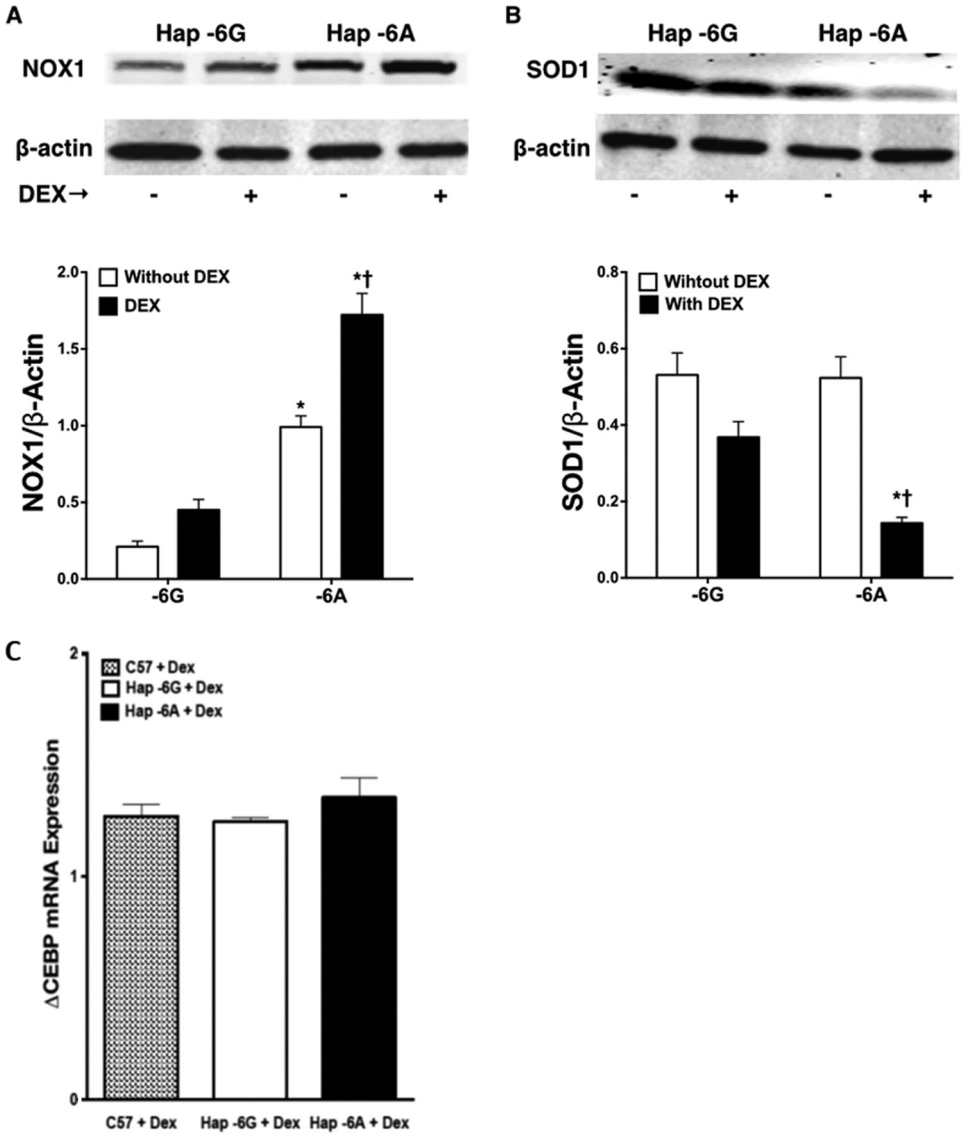

FIGURE 8. **Immunoblot analysis of redox markers in the kidney of the TG mice.** *A*, expression of NOX1; *B*, SOD1 expression. *Top panels*, representative immunoblot; *bottom panels*, densitometric analysis. *C*, hepatic CEBP mRNA expression after DEX treatment. Relative mRNA expression is calculated for each group compared with its respective untreated control. Results are shown as mean ± S.E. (*error bars*) ($n = 4$). *, $p < 0.05$ *versus* untreated Hap −6G; †, $p < 0.05$ *versus* untreated Hap −6A.

strates increased agonist-induced GR binding to these sites in the −6A Hap. DEX is a specific agonist for GR and has been widely used as a pharmacological intervention to study the effects of GR activation. Importantly, increased GR binding is not tissue-specific and is observed in both hepatic and renal tissues.

DEX-dependent increased GR binding to the hAGT promoter of the −6A Hap is reflected in significantly higher hAGT expression in these mice. Hepatic and renal tissues both exhibit higher hAGT expression. Apart from promoting stronger GR binding, we have previously shown that SNPs in the promoter of Hap −6A create a strong binding site for HNF-1α and C/EBP (3). Glucocorticoid treatment also affects the regulation of genes that contain C/EBP binding sites (19, 20), by increasing the synthesis of C/EBPβ and C/EBPδ via GR in the hepatocytes (21). Also, GR and HNF-1 act cooperatively and synergistically to increase DEX-induced expression of genes regulated by GR (22). Thus, the increased agonist-induced hAGT expression in −6A TG mice could be contributed by direct GR binding along with favorable interactions with other transcription factors, including C/EBP and HNF-1. It is noteworthy that the Hap-dependent binding of GR, preferentially to the −6A Hap, is observed in a setting of low-dose DEX treatment. The endogenous mAGT gene does not contain SNPs to modulate differential GR, HNF-1, or C/EBP binding. Contextually, we did not observe differential regulation of the mAGT gene, which lends credibility to the hypothesis of Hap-regulated GR-GRE binding of the hAGT. However, Clouston *et al.* (23) have previously shown DEX-induced up-regulation of the mAGT gene in their animal model. Their use of a high dose of DEX (20-fold higher) could partly explain the differences in results. We specifically designed our experiments to emulate pathophysiological settings where endogenous levels of glucocorticoids could be elevated in order to detect even small changes in the Hap-depen-

dent regulation of the hAGT gene. Activation of mAGT by high doses of GR agonists could involve activation of other transcription factors and indirect regulation of the gene.

DEX-induced tissue hAGT overexpression is the second key finding of the study. Importantly, this effect is brought about by a low dose of DEX. Elevated levels of plasma hAGT accompany agonist-induced increased GR binding to and subsequent hAGT induction in the liver. An immunoblotting assay for measurement of plasma hAGT level demonstrates significant DEX-induced expression of the hAGT protein in TG mice with Hap −6A, as opposed to no significant change in Hap −6G. This is not unexpected because the liver is the primary source of circulating AGT, and DEX treatment induces hAGT in the liver of the −6A TG mice. The catalytic action of renin on the AGT is essential for synthesis of decapeptide AngI that is subsequently cleaved by ACE to generate the potent effector octapeptide AngII. The classical paradigm of the RAS suggests AGT metabolism by renin as the rate-limiting step in AngII synthesis. However, an increase in blood pressure as a consequence of increased plasma AGT has been shown by Kim *et al.* (24). The concentrations of hAGT levels in the plasma are lower than the Michaelis constant (K_m) for renin (25), implying that the elevation of the plasma hAGT levels leads to an increased plasma AngII concentration. In this regard, our Western blot data show that the plasma AngII levels are significantly elevated in TG mice with Hap −6A after DEX treatment. Thus, the results underscore the significance of elevated AGT levels that in this case were brought on by differential GR regulation of the hAGT gene.

DEX-induced increased AngII in TG mice with Hap −6A leads to hypertension. The pathophysiological impact of the differential hAGT regulation by DEX is the third key finding of this study. RAS governs mean arterial pressure via AngII and aldosterone as the principal end effector molecules. Dual effects, vascular and renal, underlie the prohypertensive actions of RAS overactivity. In an acute setting, AngII causes vasoconstriction and increases the total peripheral resistance, thus increasing mean arterial pressure. Angiotensin type 1 receptor activation-dependent signaling brings about the vasoconstriction. Increased peripheral resistance, alone, is insufficient to sustain elevated mean arterial pressure for significant lengths of time because the renal pressure-natriuresis mechanism will be activated, thus promoting salt loss and reduction of elevated mean arterial pressure. However, up-regulation of renal AngII can shift the pressure-natriuresis curve to right and thus contribute to the prohypertensive effects of systemic AngII (26). In this light, significantly increased blood pressure in Hap −6A animals treated with DEX alludes to the acute effects of AngII. Additionally, DEX-induced up-regulation of the hAGT gene in the kidney of TG mice with Hap −6A may contribute toward increased blood pressure. TG mice with −6A Hap exhibit much higher GR binding, plasma hAGT, and plasma AngII after DEX treatment. A reasonable conclusion of DEX-induced, AngII-dependent hypertension can be deduced for these TG mice. The duration of DEX treatment in our study (72 h) also suggests that these prohypertensive effects are primarily of vascular origin. Increased renal AngII, by alterations in the pressure-natriuresis response, could also play a secondary role in elevating

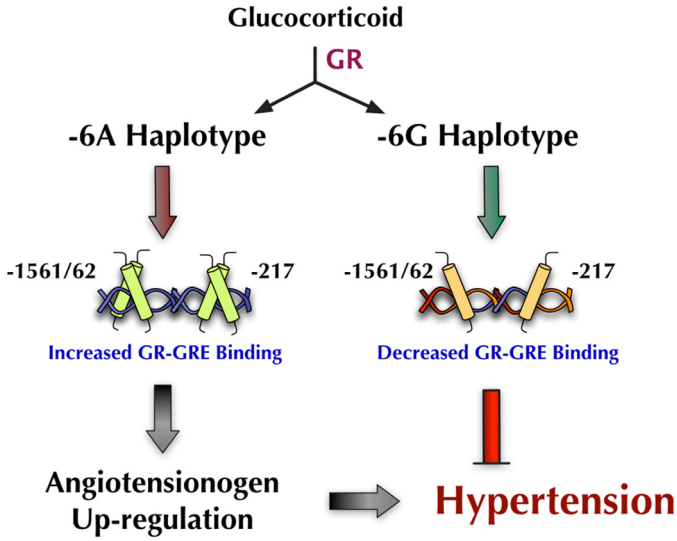

FIGURE 9. **Schematic depicting our hypothesis and conclusions; SNPs in the promoter of the hAGT gene with Hap −6A increase glucocorticoid-induced promoter activity.** Upon exposure to glucocorticoids, the activated GR binds more strongly to the promoter of the hAGT gene in TG mice with Hap −6A. This augments expression of the hAGT gene in the liver and the kidneys. Plasma AngII levels are elevated as a result of increased hAGT expression, thereby increasing the systolic blood pressure.

blood pressure in the Hap −6A TG mice. It is important to note that although Hap −6G TG mice do not show elevated plasma AngII in response to DEX treatment, they do exhibit minor elevation in blood pressure. These seemingly counterintuitive observations could be explained by nonspecific mineralocorticoid receptor activation by DEX. GR agonists activate the mineralocorticoid receptor to varying extents, and DEX is no exception. Despite these nonspecific actions of DEX, elevated plasma hAGT and AngII are the primary contributors of hypertension in −6A TG mice. These conclusions are supported by the reversal of DEX-induced hypertension in these mice by an angiotensin type 1 receptor blocker. Further evidence of the principal role of AngII in DEX-induced hypertension, in −6A TG mice, is provided by the analyses of cellular redox markers. Modulatory effects of AngII on the redox pathways are well established (27, 28). In line with reports from other investigators, we see increased NOX1 and decreased SOD1 in renal tissues from −6A animals, TG mice with DEX-induced RAS overactivity.

In conclusion, as modeled in Fig. 9, DEX treatment activates GR and modulates expression of genes containing the GRE. In this regard, SNP-directed Haps govern gene expression by modulating transcription factor binding, such as to the GR. The SNP cluster in the hAGT gene, marked by adenosine at the −6 site, predisposes to stronger GR binding by creating greater homology with the GRE consensus sequence. This provides for stronger GR-GRE interaction at these sites when challenged with an agonist. DEX is a potent GR agonist, and its treatment results in increased GR binding to −217 and −1561/62 sites of the hAGT gene containing SNPS marked by Hap −6A. Increased tissue and plasma AGT with RAS overactivity are a consequence, as are pathophysiologies like hypertension and inflammation in subjects with the −6A Hap of the AGT gene. Thus, appropriate patient screening to identify patients with

Hap −6A of the AGT gene can allay, at least partially, the increased risk of hypertension and can provide for the most effective therapeutic options targeted at reducing cardiovascular morbidity in these patients.

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