A Haplotype of Angiotensin Receptor Associated with Human Hypertension Increases Blood Pressure in Transgenic Mice

Running Title: Angiotensin Receptor Haplotype and Hypertension

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Key words: angiotensin receptor type-1, single nucleotide polymorphism, transgenic, hypertension
Background: AT\(_1\)R activation induces oxidative stress, promotes inflammation, and increases blood pressure.

Results: SNPs in AT\(_1\)R-promoter occur in linkage disequilibrium forming two haplotypes. Transgenic mice with haplotype-I have USF-2-dependent AT\(_1\)R over-expression, increased oxidative stress and blood pressure.

Conclusion: Haplotype-I leads to enhanced expression and patho-physiological effects of AT\(_1\)R.

Significance: Polymorphisms in AT\(_1\)R provide for genetic predisposition to hypertension.

The renin-angiotensin system plays an important role in the regulation of blood pressure via angiotensin II and the Angiotensin-II receptor type-1 (AT\(_1\)R). Human AT\(_1\)R gene promoter has four SNPs: T/A at -777, T/G at -680, A/C at -214, and A/G at -119 that are in linkage disequilibrium. Variants -777T, -680T, -214A, and -119A almost always occur together (named haplotype-I) and variants -777A, -680G, -214C, and -119G almost always occur together (named haplotype-II) in Caucasian subjects. Genomic DNA analyses, from 388 normotensive and 374 hypertensive subjects, links haplotype-I of the hAT\(_1\)R gene with hypertension in Caucasians (\(p = 0.004, \chi^2 = 8.46\)). Our results show increased basal promoter activity of the hAT\(_1\)R gene in cells (H295R & A7r5) transfected with reporter construct containing haplotype-I. We also show increased binding of the transcription factor, USF-2, to oligonucleotide containing nucleoside -214A as opposed to -214C. Recombineering of a 166 kb BAC containing 68 Kb of the 5’-flanking region, 45 Kb of the coding sequence and 53 Kb of the 3’-flanking region of the hAT\(_1\)R gene was employed to generate transgenic mice with either haplotype. We show that hAT\(_1\)R mRNA level is increased in the kidney and heart of transgenic mice containing haplotype-I as compared to haplotype-II; (c) blood pressure and oxidative stress is increased in transgenic mice containing haplotype-I as compared to haplotype-II.

Hypertension is a risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure (1). Renin-angiotensin system is central to the regulation of blood pressure and plays an important role in patho-physiology of hypertension (2). The octapeptide, angiotensin-II (AngII), is synthesized from its precursor molecule, angiotensinogen by sequential proteolytic action of renin and angiotensin converting enzyme (ACE). Ang II is a potent vasoactive peptide that promotes adrenal aldosterone secretion and regulates the renal excretion of salt and water (3,4). These biological responses of Ang II are mediated by its interaction with the high-affinity G-protein coupled receptors (GPCRs) localized on the surface of target cells (5). Two main Ang II receptor subtypes – the AT1 and the AT2 receptor – have been identified (6). AT1 receptor (AT\(_1\)R) is the principal mediator of the aforementioned physiological effects of Ang II in mammalian adults and mounting evidence, unequivocally, shows the contributions of AT\(_1\)R in cardiovascular pathologies (3). Ito et al have shown a 50% reduction in AT\(_1\)a receptor expression and a significantly reduced systolic blood pressure in heterozygous AT\(_1\)aR knock-out mice (7). On the other hand, Le et al (8) have generated transgenic mice over-expressing the AT\(_1\)R gene and have shown that increasing the gene copy number from 2 to 4 leads to doubling of AT\(_1\)R mRNA expression, in the vasculature and the heart of these mice. This resulted in hypertension especially in female transgenic mice. Additionally, transgenic mice overexpressing AT\(_1\)R in the cardiac myocytes demonstrate cardiac enlargement, heart failure, and early death (9,10). Spontaneously hypertensive rats (SHR) have been shown to have dys-functional AT\(_1\)R activation in the brain (11) and increased ATRs are present in the brain of DOCA-salt sensitive rats (12).
Complex cardiovascular disorders, like hypertension, are polygenic in nature with strong familial inheritance. Polymorphisms in the regulatory regions of the genes regulating blood pressure contribute to this inheritance. Contextually, the AT\(_1\)R gene is one of the prime candidates whose differential regulation may predispose to such disorders. An A/C polymorphism at position 1166 (rs5186) in the 3'-UTR of the AT\(_1\)R gene has been associated with hypertension in the Finnish (13) (14) and the Caucasian populations (15). The role of this polymorphism, however, in human hypertension remains unconfirmed. On the other hand, in Caucasian hypertensive subjects we identified four novel SNPs, in linkage disequilibrium (LD), in the promoter region of the human AT\(_1\)R (hAT\(_1\)R) gene. The SNPs are: T/A at -777, T/G at -680, A/C at -214, and A/G at -119. Variants -777T, -680T, -214A, and -119A always occur together creating haplotype-I, and variants -777A, -680G, -214C, and -119G always occur together creating haplotype-II. These SNPs may affect transcription factor binding, such as for the upstream stimulatory factor-2 (USF-2), and modulate gene expression. Thus, we generated transgenic mice (TG) with either haplotype and tested the hypothesis that differential transcriptional regulation of the hAT\(_1\)R gene will enhance hAT\(_1\)R expression and predispose to AT\(_1\)R-dependent hypertension in haplotype-I.

We show here that haplotype-I is associated with hypertension in Caucasian subjects. We also show increased promoter activity, both basal and in USF-co-transfected cells, and increased USF-2 binding to oligonucleotide corresponding to haplotype-I in adrenal (H295-R) and vascular smooth muscle (A7r5) cells. In vivo testing was done in transgenic mice containing either haplotype-I or haplotype-II of the hAT\(_1\)R gene—using recombinering of a 166 kb BAC containing 5'-flanking region, coding sequence and 3'-flanking region of the hAT\(_1\)R gene. TG mice with haplotype-I demonstrated enhanced USF-2 chromatin binding with increased hAT\(_1\)R expression in cardiac and renal tissues. Complementary experiments revealed significantly higher blood pressure; pro-inflammatory markers, including IL-6, TNF\(_\alpha\), CRP; and oxidative stress marker such as NADPH oxidase in TG mice containing haplotype-I of the hAT\(_1\)R gene as compared to TG mice with haplotype-II.

**EXPERIMENTAL PROCEDURES**

**Patient selection:** We have analyzed the genomic DNA from 374 hypertensive (182 males and 192 females), (mean age: 59 ±10 years) and 388 normotensive subjects (182 males and 206 females), (mean age 58 ±10 years). All of these subjects were recruited from the outpatient department of Westchester Medical Center, Valhalla, NY or from AmDec foundation. All case and control subjects gave informed consent before participating in the research. The research protocol was approved by the Institutional Review Board at New York Medical College. Individuals were excluded if they had a previous history of coronary artery disease, peripheral vascular disease, cerebrovascular disease, secondary hypertension, diabetes mellitus and renal diseases. For a period of 30 min before BP measurement, no exercise, alcohol, caffeine, or smoking were allowed. BP was measured by conventional mercury sphygmomanometer. Measurements by two different observers were taken at the left arm with individuals in the seated position after 15 min of resting. The criteria for hypertension was defined as a systolic blood pressure (SBP) > 140 mmHg, a diastolic blood pressure (DBP) > 90 mmHg, or under antihypertensive therapy. The normotensives (with SBP/DBP < 140/90 mmHg) without a history of hypertension and without diabetes mellitus were recruited from the same population and matched for the sex and age. Clinical data of the patients was self reported during a detailed pre-study examination and confirmed by medical charts provided by their physicians.

**Analysis of Genomic DNA:** The genomic DNA was amplified using 5'-TGT AAA CTA CAG TCA CCC TCT CAC C-3' as a forward primer and 5'-ATG AGG CAG TAT CAC CCT GA-3' as a reverse primer to amplify 238 bp 5'-flanking region of the hAT\(_1\)R gene containing the T/G polymorphic site at -680
position of the promoter. The amplified fragment was then analyzed by 3.5% agarose gel electrophoresis after Hinf1 restriction digestion. Hinf1 recognizes the nucleotide sequence GANTC and cleaves the amplified DNA when nucleoside A is present at -680 to produce 127 and 111 bp fragments. The results obtained by restriction analysis were confirmed by direct sequencing of representative amplified fragments.

**Plasmid construction:** The reporter constructs pHAT1Rhap-1luc (Haplotype-I) and pHAT1Rhap-2luc (Haplotype-II) were synthesized by PCR amplification of the human AT1R gene using TGTAGGCTTTGTCCATTTTT as the forward primer and GTCCAGACGTCTCCTCCTCTGAG as the reverse primer using DNA from human subjects containing either Haplotype-I or Haplotype-II of the hAT1R gene. The amplified fragment contained nucleotides -1174 to +55 and was subcloned in the pGL3-basic vector lacking eukaryotic promoter and enhancer sequences (Promega). Expression vector RSV-β-gal was obtained from Promega. Plasmid DNAs for transient transfection were prepared by Qiagen midi or maxi plasmid kits (Chatsworth, CA) using conditions described by the manufacturer.

**Cell culture and transient transfection:** Human adrenocortical carcinoma cells (H295-R) or vascular smooth muscle cells (A7r5) were grown as monolayers in DMEM-F12 medium (Gibco) supplemented with 20% Nu-Serum IV, 1% ITS (BD Biosciences), 100 units/ml penicillin, and 100μg/ml streptomycin in an atmosphere of 5% CO2. For transient transfections, reporter DNA (500 ng) and β-galactosidase DNA (10ng) were mixed with pBluescript DNA to a final weight of 1μg of DNA. Transient transfections were performed with Fugene 6 transfection reagent (Roche) following the manufacturer’s protocol. For cotransfection experiments, expression vector pSV-USF-2 (100ng) was added to the reporter constructs. After 24h of transfection, cells were serum starved for an additional 24h. Cells were harvested 48h post-transfection, and whole cell extracts were prepared by resuspension in 200μl of lysis buffer (Promega). An aliquot of the cell extract was used to measure luciferase activity in a Turners Design 20/20 luminometer using a luciferase assay system (Promega) as described by the manufacturer. Luciferase activity was normalized to β-galactosidase activity that was determined using the β-glo assay system (Promega).

**Gel Mobility Shift Assay:** The probes for electrophoretic mobility shift assay were chemically synthesized by MWG Biotech (High Point, NC), annealed and radiolabeled at the 5’-ends by polynucleotide kinase using [γ-32P]ATP. DNA fragments (100,000cpm), poly(dI-dC) (2μg), BSA (20μg), and nuclear extract (5-10μg) were incubated in a solution containing 25mM HEPES (pH 7.5), 100mM NaCl, 0.2mM PMSF, 2mM dithiothreitol, and 5% glycerol on ice for 30min and separated on a 6% polyacrylamide gel in a cold room. After 2-3h, the gel was dried under vacuum, and protein-nucleic acid complexes were identified by autoradiography. For supershift assay 5μg of USF-1 antibody (Santa Cruz Biotechnology) was added to the reaction mixture, which was incubated for 20min and analyzed by electrophoretic mobility shift assay. Radioactive nucleotides were purified by chromaspin columns (BD Biosciences). Nuclear extracts for gel mobility shift assays were prepared by modification of a previously described method (16).

**Oligonucleotides:** Double-stranded oligonucleotides 214A and 214C were obtained by annealing CGGGACCACGTGAAC, and CGGGACCACGTGAAC with their respective complementary oligonucleotides. Double-stranded oligonucleotide MLTF containing the consensus USF binding site was obtained by annealing CTAGTGAGCAGCCAGTGACGG with its respective complementary oligonucleotide (USF binding site is underlined). Oligonucleotides containing consensus HNF-3 binding site was obtained by annealing CTAGTATTATTGACTAGAT with its complementary oligonucleotide.
Animal Experiments: Procedures were approved by the University of Toledo Animal Care and Use Committee and performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH Publication No. 85-23, revised 1996).

Preparation and modification of hAT1R BAC: BAC clone (CTD-3080C3) containing hAT1R gene was purchased from CalTech. The plasmid was grown in low salt LB medium containing 12.5µg/ml chloramphenicol, at 37°C, and BAC DNA was purified using Nucleobond BAC maxiprep kit (BD Biosciences). The promoter sequence in the wild type hAT1R BAC was modified by recombineering using the galK 2-step procedure in SW102 cells (17). SW102 cells were grown at 32°C. First, the 1.7 Kb of promoter region of the hAT1R gene was deleted by inserting galK cassette. Next, the galK cassette was replaced with a 1.7 Kb promoter region from the DNA of a subject containing mutant haplotype of the hAT1R gene. Wild-type and modified BAC DNAs were analyzed by fingerprinting after treatment with SpeI (releasing 30 fragments varying in size from 500bp to 16kb). The modified region of the hAT1R BAC was finally confirmed by direct sequencing. The nucleotide sequence of the promoter, all the exons and 3’-flanking region were sequenced to confirm that the two BAC differ only in the promoter region and contained either haplotype-I or haplotype-II of the hAT1R gene.

Generation of Transgenic mice, containing Haplotype-I and Haplotype-II of the hAT1R gene: In order to understand the regulation of human AT1R gene in an in vivo situation, we generated transgenic mice using 166kb BAC plasmid DNA containing hAT1R gene. This BAC contains 68kb of the 5’ flanking region, 45kb of the coding region and 53kb of the 3’ flanking region of the hAT1R gene cloned in 7.4kb of pBeloBAC11 vector (Fig. 6A). We characterized the BAC by restriction digestion with SpeI enzyme followed by gel electrophoresis. This treatment produced 30 DNA fragments varying in size from 500bp to 16kb as predicted (Fig. 6B). Nucleotide sequence of the BAC revealed that it contains Haplotype-I of the hAT1R gene. The 1.2 Kb promoter of the hAT1R gene in this BAC was modified to Haplotype-II by re-combineering using 1.2Kb promoter fragment from a human subject containing haplotype-II of the hAT1R gene as described previously for hAGT gene (18). The nucleotide sequence of the resulting BAC was confirmed by sequence analysis. The linearized DNA fragments from the wild type and recombineered BACs were obtained after NotI treatment and were used for microinjection to produce transgenic mice. The presence of hAT1R gene was analyzed by PCR amplification of tail DNA of transgenic mice. The location of different regions of the BAC plasmid that were used for PCR amplification to confirm the integration of hAT1R gene in mouse genome is shown in Fig.6C. Nucleotide sequence of primers used for the amplification of different regions of hAT1R gene in transgenic animals is presented in Table-I. Initially we obtained three transgenic lines from each construct but after characterization we maintained only one TG line for each haplotype of hAT1R gene. These TG mice have single copy of the hAT1R gene as determined by Q-PCR (19).

Q-RT-PCR for mRNA analysis: Kidney and heart from 10-12 weeks old hAT1R transgenic male mice were harvested and immediately frozen in liquid nitrogen for storage at -80°C. RNA was isolated using RNeasy Plus mini kit (Qiagen). RNA (1µg) was reverse-transcribed into cDNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas). QRT-PCR was performed using primers for human AT1R, IL6, TNFα, and CRP from SAB using standard protocol. Relative transcript abundance was calculated following normalization with mouse GAPDH.

In vivo ChiP Assay: The chromatin immunoprecipitation (ChiP) assay was performed using the EZ ChiP Chromatin Immunoprecipitation kit from Millipore (CA, USA). Mice were perfused with normal saline. Kidney was excised and washed in PBS (phosphate buffered saline); minced into smaller pieces; fixed with 1% formaldehyde for 20min at room temperature; washed with chilled PBS followed by their lysis. The DNA
was fragmented by sonication and 10μl of the chromatin solution was saved as input. A 5μg amount of USF2 or RNA pol II antibody or rabbit immunoglobulin G was added to the tubes containing 900μl of sonicated chromatin solution, and the mixture was incubated overnight at 4ºC. The antibody complexes were captured with protein A-agarose beads and subjected to serial washes (as described in manufacturer’s protocol). The chromatin fraction was extracted with SDS buffer and reverse cross-linked at 65ºC for 4-6h. The DNA was then purified using Qiagen miniprep column. The immuno-precipitated DNA (1μl) and the input DNA (1μl) were subjected to 35 cycles of PCR amplification using (a) -213for TCCGACAGGATACTCC as a forward and -213rev ACGAGGTCTCAGTTTGCATT as a reverse primer when USF2 antibody was used for immunoprecipitation. This amplified 217 bp fragment spanning the USF binding site located around -213 region of the human AT1R gene promoter (b) -119for TGATAGTTGACACGGGACGA as a forward primer and -119rev TTTATAGTGAGGGCGTTGC as a reverse primer when RNA pol II antibody was used. This amplified 178bp fragment containing RNA pol II binding region of the human AT1R gene promoter. The PCR amplified products were analyzed on 2% agarose gel.

**Blood Pressure Measurement in Transgenic Mice:** All mice were fed standard mice chow and had access to water ad libitum. Blood pressure (BP) was measured in the conscious state by Radio telemetry as described previously (20). After 4 days of basal blood pressure measurement, animals were treated with AT1R blocker, losartan (Sigma Aldrich, USA) (30mg/kg/day), in drinking water as described previously (18). The blood pressure was then measured 24h after losartan treatment. Mean BP values were calculated for every hour from the values taken over 24h. Animals were housed in individual cages.

**Statistical Analysis:** The Graphpad statistical software package (Graphpad 3.00 for Windows, Graphpad Software, San Diego, CA) was used for calculating the differences between allele frequencies at different times. Statistical significance was assessed using ANOVA with Tukey-Kramer Post hoc test. Statistical significant results are marked by an asterisk (p<0.05).

**RESULTS**

-680T allele of the hAT1R gene is associated with increased risk of hypertension in Caucasians: Due to LD of the SNPs in the second haplotype, -680T or -680G was used as reporter polymorphism for haplotype I and II respectively (Fig.1). DNA from 374 hypertensive and 388 normotensive subjects was analyzed by restriction analysis followed by gel electrophoresis (Fig.2). -680T allele of the hAT1R gene is associated with hypertension in Caucasian subjects (p = 0.004, \( \chi^2 = 8.46 \)) (allele frequency of 680T in hypertensive subjects was 0.80 and in normotensive subjects was 0.74). All patients and control subjects were in Hardy-Weinberg equilibrium. The results of this analysis suggest that -680T allele of the AT1R gene to be a risk factor for hypertension in Caucasian subjects (Table-I).

**Reporter construct containing haplotype-I of the hAT1R gene has increased promoter activity as compared to the reporter construct containing haplotype-II on transient transfection:** Reporter constructs with 1.2Kb region of the hAT1R gene promoter of haplotypes I and II were attached in front of the luciferase gene in the basic vector pGL3-luc. We next performed transient transfections of the reporter constructs pHAT1Rhap-1 regulatory (Haplotype-I) and pHAT1Rhap-2 regulatory (Haplotype-II) in H295R cells. These cells have been shown to contain functional angiotensin-II receptors (21). Our results show ~4 fold increased (p<0.05) promoter activity of the hapltype-I reporter construct as compared to haplotype-II (Fig.3A).
cells. AT1R gene is expressed in these cells, which were transfected with either pHA1Rhap-1lac or pHA1Rhap-2lac. Increased promoter activity of the reporter construct with haplotype-I, ~1.9 fold (p<0.05), was observed as opposed to the promoter activity of haplotype-II (Fig.3B).

**Nucleotide sequence of hAT1R gene promoter containing -214A binds strongly to transcription factor USF as compared to -214C:** The USF consensus sequence, CANNTG, exhibits greater sequence homology to the hAT1R promoter of haplotype-I when compared to that of haplotype-II. Site -214 is the principal determinant of this preference, where -214A of haplotype-I could provide for stronger USF binding than -214C of haplotype-II (Fig. 4A). The hypothesis was tested with gel shift assay performed using radioactive oligonucleotides -214A and -214C as probes and H295R extract as a source of transcription factors. Competition was then performed in the presence of cold oligonucleotides 214A, 214C, consensus USF site, non-specific oligonucleotide containing HNF-3 binding site, and in the presence or absence of USF-1 antibody (Fig. 4B). Radioactive oligonucleotide 214A formed a complex (shown by an arrow) (lane 1) that was competed out with self-oligonucleotide (lanes 2, 3) or an oligonucleotide-containing consensus USF binding site (lane 6) but not with 214C or non-specific oligonucleotides (lanes 4, 5, 8). This complex was also removed in the presence of USF-1 antibody (lane 7). On the other hand, radioactive oligonucleotide 214C did not form this complex but formed a faster moving complex (lane 9). These results allude to a stronger USF binding to haplotype-I of the hAT1R gene.

**USF-2 increases the promoter activity of reporter construct containing haplotype-I of the hAT1R gene, as compared to haplotype-II in H295R cells:** In line with our earlier results, we examined effects of USF on promoter activity of constructs with either haplotype. Expression vector containing coding region of the USF2 was co-transfected with reporter constructs hAT1Rluc1 hap-1 or hAT1Rluc2 hap-2 in H295R cells. USF2 increased (p<0.05) the promoter activity of reporter construct containing haplotype-I as compared to haplotype-II of the hAT1R gene (results are expressed as fold increase relative to basal promoter activity) (Fig.5).

**TG mice with haplotype-I show increased hAT1R expression, in renal and cardiac tissues, as compared to TG mice with haplotype-II:** Haplotype-dependent, physiological regulation and role of the hAT1R gene was assessed in TG mice generated with each haplotype. The AT1R gene is not uniformly expressed in all mammalian tissues, with high baseline expression in the renal and cardiac tissues. To preserve this tissue-specific regulation of the transgenic gene, BAC containing 68kb of the 5' flanking region, 45kb of the coding region and 53kb of the 3' flanking region of the hAT1R gene was used to generate TG mice (Fig. 6). Q-RT-PCR analysis was performed to quantify hAT1R expression in renal and cardiac tissues of the two haplotypes. Our results show increased, renal (~6 fold, p<0.05) and cardiac (~3 fold, p<0.05), hAT1R gene expression in TG mice with haplotype-I as compared to ones with haplotype-II (Fig. 7A & B). Complementary experiments show no significant difference in the mRNA levels of the mAT1R in these tissues (data not shown).

**USF2 and RNA polymerase have higher affinity to the chromatin of kidney from TG mice containing haplotype-I of the hAT1R gene as compared to haplotype-II:** In vitro results, where USF2 increased promoter activity of the reporter construct containing 1.2 Kb promoter with haplotype-I of the hAT1R gene, were confirmed in vivo by ChIP-analysis. As shown in Fig.8 (A & C), a 2.2-fold increase (p<0.05) in USF2-binding is seen to the nucleotide sequence around -214-region of the chromatin from TG mice containing haplotype-I. As hAT1R mRNA level is increased in the kidneys of TG animals containing haplotype-I of the hAT1R gene, we also performed a ChIP assay using antibody against RNA polymerase-II. Results of this experiment showed that RNA polymerase-II has stronger affinity to the nucleotide sequence present in the chromatin of...
transgenic animals containing haplotype-I of the hAT1R gene as compared to haplotype-II by 1.6 fold (Fig. 8B & D).

Expression of pro-inflammatory and oxidative markers is increased in the kidney of transgenic animals containing haplotype-I as compared to haplotype-II of the hAT1R gene: Functional relevance of the up-regulated hAT1R was assessed by expression analysis of the pro-inflammatory/oxidative markers, including IL6, TNFα, CRP, and NADPH-oxidase. ANGII, via AT1R-activation, induces synthesis and secretion of these inflammatory cytokines. Renal homogenates show an increase in the mRNA levels of IL6 (2.05 fold), TNFα (2.8 fold), and CRP (2.2 fold) in the kidney of male hAT1R-TG mice containing haplotype-I as compared to haplotype-II. (Fig. 9A, p<0.05). Complementary experiments show up-regulation of NADPH-oxidase mRNA (Fig. 9B) in the kidneys of male hAT1R TG mice containing haplotype-I as compared to haplotype-II (p<0.05).

Blood Pressure is increased in Transgenic Mice containing haplotype-I of the hAT1R gene as compared to mice containing haplotype-II: Increased AT1R density is expected to modulate blood pressure by sensitizing effector organs to the effects of circulating ANGII. Blood pressure recordings, using telemetry-probes, were obtained in both haplotypes of TG-mice. Systolic blood pressure (SBP) was measured over a period of four days in 24hr durations. As shown in Fig. 10A, mean SBP of TG-mice containing haplotype-I was significantly higher (p<0.05) from that in TG-mice containing haplotype-II. To confirm the role of increased hAT1R in bringing about this difference in SBP, experiments were conducted in the absence and in the presence of an AT1R-selective blocker (losartan). In animals treated with losartan the haplotypic-difference in SBP was not observed (Fig. 10B).

Discussion
ANGII activates AT1R and regulates the mean arterial pressure via vascular and renal effects. This study, for the first time, demonstrates an increased prevalence of a haplotype characterized by AT1R-over expression in hypertensive-Caucasians. The first key finding of the study identifies two distinct haplotypes of the human AT1R-gene with four SNPs (-777T/A, -680T/G, -214A/C, and -119A/G) always occurring in LD. Haplotype-I, with TTAA at these positions, occurs with a significantly higher frequency in hypertensive subjects than haplotype-II. This is the first report characterizing these haplotypes and demonstrating haplotype-dependent association with human hypertension. This association is accompanied by increased AT1R expression in adrenal and vascular smooth muscle cells transfected with haplotype-I. Increased promoter activity of haplotype-I, when compared to haplotype-II, is the causative factor for AT1R overexpression in this haplotype.

The nucleotide sequence of the hAT1R promoter containing nucleoside A at -214 has greater homology with E-box (CANNTG) that is recognized by helix-loop-helix (HLH) family of transcription factors (22). Upstream stimulatory factor-1 (USF1) is a member of the basic helix–loop–helix leucine zipper (bHLH-zip) family of transcription factors (23). USF1 is a ubiquitous transcription factor which was originally discovered by its ability to bind to the adenovirus major late promoter and regulate its expression. USF1 binds as a homo-dimer, or as a heterodimer with the related transcription factor USF2 to E boxes and controls the expression of a number of genes (24,25). USF has been shown to increase the expression of genes involved in glucose and lipid metabolism (26). Overexpression of USF2 in transgenic mice influences metabolic traits such as obesity, lipid profiles, and glucose/insulin ratio (27). Polymorphisms in the USF1 gene have been identified in human linkage studies with diabetic nephropathy (28). Additionally, earlier studies have established SNP-dependent differential gene-regulation by USF. In this regard, we have reported SNP in USF1-binding sites in the
hAGT gene expression in adipose tissue (30). Genes, including the c-reactive protein, also encode two SNPs that modulate USF binding and account for differences in baseline CRP levels in apparently healthy people (31). In line with these studies, we show here increased USF binding to the -214A site corresponding to haplotype-I, as compared to -214C site analogous to haplotype-II. Complementary studies confirm USF-dependent up-regulation of hAT_1R expression in cells co-transfected with USF and hAT_1R. Since USF is the only transcription factor that differentially binds to -214A and -214C alleles of the hAT_1R gene, we have analyzed its role in transcriptional regulation in the present communication. However, the role of other transcription factors that may interact with USF or bind to other polymorphic sites remains to be examined.

A homologous set of transcription factors in mice and humans provides for a unique opportunity to study individual human gene regulation in an in vivo setting. The second key finding of the study demonstrates haplotype-specific regulation of the hAT_1R gene in TG mice, where haplotype-I favors increased expression of hAT_1R in renal and cardiac tissues of these mice. Absence of such a difference in the expression of mAT_1R points to the haplotype-dependent differential regulation of the hAT_1R. Complementary experiments show increased USF and RNA polymerase II binding to chromatin extracts from renal tissues of TG mice with haplotype-I. These results are in agreement with our in vitro findings and implicate USF-directed enhanced transcription of the hAT_1R gene in bringing about higher hAT_1R expression in haplotype-I vs. haplotype-II. This is the first report citing haplotype-dependent differences in expression of the hAT_1R gene in TG mice, where genetic constitution of the mice with two haplotypes is the same except for the 1.2Kb promoter region containing the aforementioned SNPs. This provides for a strong argument for USF-mediated up-regulation of the hAT_1R gene whose SNP-defined haplotypes favor differential binding of the transcription factors and promotes gene expression in one haplotype over the other.

The physiological significance of these haplotypes, and their derivative differential regulation of hAT_1R expression, is provided by the examination of the end effects of hAT_1R activation. In this regard, the third key finding of the study shows elevation of the systolic blood pressure and development of a pro-inflammatory/pro-oxidative state in TG mice with increased expression of hAT_1R. AT_1R is a GPCR and is ubiquitously expressed in mammalian tissues. ANGII-mediated AT_1R-dependent physiological effects of the RAS include regulation of renal salt and water balance, vascular tone, and cardiac structure and contractility. Together, these variables govern long-term set point for the mean arterial pressure. Additionally, chronic over-activation of AT_1R promotes cellular oxidative stress, primarily via activation of NADPH oxidase, and secretion of pro-inflammatory/pro-fibrotic cytokines (for a review see Ref (32)). Increased expression of cytokines, including IL-6, TNFα, and CRP; and increased levels of NADPH oxidase component, NOX1, confirm the functional role of increased hAT_1R activation in TG mice with haplotype-I. Both mAT_1R and hAT_1R are amenable to activation by mouse ANGII and the haplotype-dependent differential effects in measured physiological parameters corroborate the contributions of increased hAT_1R density in TG mice with haplotype-I. Higher AT_1R expression will allow for more spare receptors, thus sensitizing the tissues to circulating levels of ANGII. Elevation of the SBP in TG mice with haplotype-I supports this notion. Reversal of this elevated SBP by an AT_1R-blocker is supportive of earlier evidence linking increased AT_1R expression to heightened patho-physiological response to endogenous RAS. Since our main objective was to examine the effect of these haplotypes of the human AT_1R gene on its expression and to understand their role in blood pressure regulation, we did not use wild type C57 animals as control.

In conclusion, we provide the first evidence linking human hypertension with haplotype-dependent variability in AT_1R.
expression. We provide *in vitro* and *in vivo* evidence of USF-mediated transcriptional up-regulation of the hAT,R gene in haplotype-I, as opposed to haplotype-II. Finally, our results showing increased tissue expression of inflammatory/oxidative markers and an elevated SBP in haplotype-I provide pathophysiological perspectives to the study. Where chronic activation of RAS is frequently associated with cardiovascular and renal abnormalities, as seen in animals with Goldblatt’s hypertension, increased AT,R expression has the potential to bring about similar patho-physiological alterations. This study provides evidence for the notion that genetic susceptibility towards increased AT,R-density will alter RAS-regulated physiological systems by sensitizing them to endogenous levels of ANGII, which may or may not be elevated. We are currently pursuing detailed physiological studies to identify the impact of these haplotypes on cardiac, vascular, and renal function.

Reference List

1. Galis, Z. S., Thrasher, T., Reid, D. M., Stanley, D. V., and Oh, Y. S. (2013) Investing in high blood pressure research: a national institutes of health perspective *Hypertension* **61**, 757-761

2. Griendling, K. K., Murphy, T. J., and Alexander, R. W. (1993) Molecular biology of the renin-angiotensin system *Circulation* **87**, 1816-1828

3. Griendling, K. K., Ushio-Fukai, M., Lassegue, B., and Alexander, R. W. (1997) Angiotensin II signaling in vascular smooth muscle. New concepts *Hypertension* **29**, 366-373

4. Peach, M. J. and Dostal, D. E. (1990) The angiotensin II receptor and the actions of angiotensin II *J. Cardiovasc. Pharmacol*. **16 Suppl 4**, S25-S30

5. Mendelsohn, F. A. (1985) Localization and properties of angiotensin receptors *J. Hypertens.* **3**, 307-316

6. Timmermans, P. B., Wong, P. C., Chiu, A. T., Herblin, W. F., Benfield, P., Carini, D. J., Lee, R. J., Wexler, R. R., Saye, J. A., and Smith, R. D. (1993) Angiotensin II receptors and angiotensin II receptor antagonists *Pharmacol. Rev.* **45**, 205-251

7. Ito, M., Oliverio, M. I., Mannon, P. J., Best, C. F., Maeda, N., Smithies, O., and Coffman, T. M. (1995) Regulation of blood pressure by the type 1A angiotensin II receptor gene *Proc. Natl. Acad. Sci. U. S. A* **92**, 3521-3525

8. Le, T. H., Kim, H. S., Allen, A. M., Spurney, R. F., Smithies, O., and Coffman, T. M. (2003) Physiological impact of increased expression of the AT1 angiotensin receptor *Hypertension* **42**, 507-514

9. Hein, L., Stevens, M. E., Barsh, G. S., Pratt, R. E., Kobilka, B. K., and Dzau, V. J. (1997) Overexpression of angiotensin AT1 receptor transgene in the mouse myocardium produces a lethal phenotype associated with myocyte hyperplasia and heart block *Proc. Natl. Acad. Sci. U. S. A* **94**, 6391-6396
10. Paradis, P., Dalí-Youcef, N., Paradis, F. W., Thibault, G., and Nemer, M. (2000) Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling *Proc. Natl. Acad. Sci. U. S. A* 97, 931-936

11. Veerasingham, S. J. and Raiizada, M. K. (2003) Brain renin-angiotensin system dysfunction in hypertension: recent advances and perspectives *Br. J. Pharmacol.* 139, 191-202

12. Gutkind, J. S., Kurihara, M., and Saavedra, J. M. (1988) Increased angiotensin II receptors in brain nuclei of DOCA-salt hypertensive rats *Am. J. Physiol* 255, H646-H650

13. Perola, M., Kainulainen, K., Pajukanta, P., Terwilliger, J. D., Hiekkalinna, T., Ellonen, P., Kaprio, J., Koskenvuo, M., Kontula, K., and Peltonen, L. (2000) Genome-wide scan of predisposing loci for increased diastolic blood pressure in Finnish siblings *J. Hypertens.* 18, 1579-1585

14. Kainulainen, K., Perola, M., Terwilliger, J., Kaprio, J., Koskenvuo, M., Syvanen, A. C., Vartiainen, E., Peltonen, L., and Kontula, K. (1999) Evidence for involvement of the type 1 angiotensin II receptor locus in essential hypertension *Hypertension* 33, 844-849

15. Bonnardeaux, A., Davies, E., Jeunemaitre, X., Fery, L., Charru, A., Clauser, E., Tiret, L., Cambien, F., Corvol, P., and Soubrier, F. (1994) Angiotensin II type 1 receptor gene polymorphisms in human essential hypertension *Hypertension* 24, 63-69

16. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei *Nucleic. Acids. Res.* 11, 1475-1489

17. Warming, S., Costantino, N., Court DL, Jenkins, N. A., and Copeland, N. G. (2005) Simple and highly efficient BAC recombineering using galK selection *Nucleic Acids Res.* 33, e36

18. Jain, S., Tillinger, A., Mopidevi, B., Pandey, V. G., Chauhan, C. K., Fiering, S. N., Warming, S., and Kumar, A. (2010) Transgenic mice with -6A haplotype of the human angiotensinogen gene have increased blood pressure compared with -6G haplotype *J. Biol. Chem.* 285, 41172-41186

19. D'haene, B., Vandesompele, J., and Hellemans, J. (2010) Accurate and objective copy number profiling using real-time quantitative PCR *Methods* 50, 262-270

20. Butz, G. M. and Davisson, R. L. (2001) Long-term telemetric measurement of cardiovascular parameters in awake mice: a physiological genomics tool *Physiol Genomics* 5, 89-97

21. Bird, I. M., Mason, J. L., and Rainey, W. E. (1994) Regulation of type 1 angiotensin II receptor messenger ribonucleic acid expression in human adrenocortical carcinoma H295 cells *Endocrinology* 134, 2468-2474

22. Massari, M. E. and Murre, C. (2000) Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms *Mol. Cell Biol.* 20, 429-440

23. Sawadogo, M. and Roeder, R. G. (1985) Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region *Cell* 43, 165-175
24. Sawadogo, M. (1988) Multiple forms of the human gene-specific transcription factor USF. II. DNA binding properties and transcriptional activity of the purified HeLa USF. J. Biol. Chem. 263, 11994-12001

25. Sirito, M., Lin, Q., Maity, T., and Sawadogo, M. (1994) Ubiquitous expression of the 43- and 44-kDa forms of transcription factor USF in mammalian cells. Nucleic. Acids. Res. 22, 427-433

26. Vallet, V. S., Casado, M., Henrion, A. A., Bucchini, D., Raymondjean, M., Kahn, A., and Vaulont, S. (1998) Differential roles of upstream stimulatory factors 1 and 2 in the transcriptional response of liver genes to glucose. J. Biol. Chem. 273, 20175-20179

27. Wu, S., Mar-Heyming, R., Dugum, E. Z., Kolaitis, N. A., Qi, H., Pajukanta, P., Castellani, L. W., Lusis, A. J., and Drake, T. A. (2010) Upstream transcription factor 1 influences plasma lipid and metabolic traits in mice. Hum. Mol. Genet. 19, 597-608

28. Ewens, K. G., George, R. A., Sharma, K., Ziyadeh, F. N., and Spielman, R. S. (2005) Assessment of 115 candidate genes for diabetic nephropathy by transmission/disequilibrium test. Diabetes 54, 3305-3318

29. Zhao, Y. Y., Zhou, J., Narayanan, C. S., Cui, Y., and Kumar, A. (1999) Role of C/A polymorphism at -20 on the expression of human angiotensinogen gene. Hypertension 33, 108-115

30. Dickson, M. E., Tian, X., Liu, X., Davis, D. R., and Sigmund, C. D. (2008) Upstream stimulatory factor is required for human angiotensinogen expression and differential regulation by the A-20C polymorphism. Circ. Res. 103, 940-947

31. Szalai, A. J., Wu, J., Lange, E. M., McCrory, M. A., Langefeld, C. D., Williams, A., Zakharkin, S. O., George, V., Allison, D. B., Cooper, G. S., Xie, F., Fan, Z., Edberg, J. C., and Kimberly, R. P. (2005) Single-nucleotide polymorphisms in the C-reactive protein (CRP) gene promoter that affect transcription factor binding, alter transcriptional activity, and associate with differences in baseline serum CRP level. J. Mol. Med. 83, 440-447

32. Mehta, P. K. and Griendling, K. K. (2007) Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am. J. Physiol Cell Physiol 292, C82-C97

FOOTNOTES

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FIGURE LEGENDS

Fig.1: Nucleotide sequence of Haplotype-I and II of the hAT\textsubscript{1}R gene. Human AT\textsubscript{1}R gene contains four polymorphic sites in its promoter that are in linkage disequilibrium (LD).
Haplotype-I contains nucleoside T, T, A, A at -777, -680, -214, and -119 respectively in its promoter. Haplotype-II contains nucleoside A, G, C, G at these positions.

**Fig. 2:** _Restriction analysis to determine the frequency of haplotype-I and II of the hAT$_1$R gene._ Since four polymorphisms in the hAT$_1$R promoter are in LD, the frequency of each haplotype may be calculated by measuring frequency of any polymorphism. Restriction enzyme _Hinf-I_ digest the PCR amplified fragment only when -680T is present. Therefore, genomic DNA from hypertensive patients and normotensive controls was amplified to produce 238-bp fragments as described under Experimental Procedures. The partial nucleotide sequence of the amplified fragment around the T/G polymorphic site at -680 of the AT$_1$R gene is shown in the _first line_. The _Hinf I_ restriction site (which will cleave the amplified DNA if nucleoside T is present at -680) is shown in the _second line_. The amplified DNA fragments were treated with _Hinf I_ and separated on a 3.5% agarose gel. _Lanes 1 and 6_, samples from TT homozygotes; _Lanes 2, 4, and 5_, samples from GT heterozygotes; _Lane 3_, sample from a GG homozygote; _Lane 7_, positions of DNA markers.

**Fig. 3:** _Basal promoter activity of reporter constructs containing haplotype-I of the human AT$_1$R gene promoter is increased in H295R (A) and A7r5 (B) cells as compared to haplotype-II._ Basal promoter activity of reporter constructs containing either haplotype-I or II was determined by transient transfection in H295R or A7r5 cells. Relative luciferase activity of the reporter construct containing haplotype-I (solid bar) was calculated by assuming the luciferase activity of the reporter construct containing haplotype-II (empty bar) as 1. The luciferase activity of each construct is the average of three sets of triplicate experiments, error bars are standard error of the mean (SEM). *p<0.05 vs. Haplotype-II

**Fig. 4:** A: _Nucleotide Sequence of hAT$_1$R promoter containing -214A has stronger homology with USF binding site B: Oligonucleotide containing -214A binds strongly to USF as compared to the oligonucleotide containing -214C._ Oligonucleotide 214A formed a complex (shown by an arrow) (lane 1) which was competed out with self oligonucleotide (lanes 2, 3), USF consensus oligonucleotide (lane 6), but not with an oligonucleotide containing -214C (lanes 4, 5) or a non-specific oligonucleotide (lane 8). This complex was also removed in the presence of USF-1 antibody (lane 7). On the other hand, radioactive oligonucleotide 214C did not form this complex but formed a faster moving complex (lane 9).

**Fig. 5:** _Co-transfection of USF2 increases the promoter activity of reporter construct containing haplotype-I of the hAT$_1$R gene as compared to haplotype-II on transient transfection in H295R cells._ Reporter constructs containing either haplotype-I or haplotype-II of the hAT1R gene was co-transfected with an expression vector containing USF2 coding region and luciferase activity was determined after transient transfection in H295R cells. Luciferase activity of reporter construct containing haplotype-I is shown by solid bar and that of haplotype-II by empty bar. The promoter activity after co-transfection of expression vectors was calculated by assuming the basal promoter activity (control) as one. The luciferase activity of each construct is the average of three sets of triplicate experiments, error bars are SEM. *p<0.05 vs. Haplotype-II

**Fig. 6:** _Generation of Transgenic mice containing haplotype-I and II of the hAT$_1$R gene:_ Transgenic mice were generated using 166kb BAC plasmid DNA containing hAT$_1$R gene by recombineering as described in Methods and Materials. A: Diagrammatic presentation of the 5’-UTR, coding region, and 3’-UTR of hAT$_1$R gene of BAC construct in 7.4kb of pBeloBAC11 vector. B: Restriction digestion of BAC containing hAT$_1$R gene with restriction enzyme Spe1 followed by gel electrophoresis was performed to determine authenticity of the BAC. Lane 1
shows the molecular size marker of 1kb ladder DNA; lanes 2 and 3 show DNA fragments obtained from the restriction digestion of haplotype-I and haplotype-II respectively. C: location of primers designed from different regions of the BAC plasmid for the characterization of hAT1R gene.

Fig. 7: hAT1R mRNA shows increased expression in transgenic mice containing haplotype-I: Relative mRNA levels of hAT1R were analyzed from the kidney (A) and heart (B) of transgenic mice containing haplotype-I and haplotype-II of the hAT1R gene using QRT-PCR. Results were normalized to mouse GAPDH. Error bars are SEM (n=6). *p<0.05 vs. Haplotype-II

Fig. 8: Chromatin Immunoprecipitation Assay shows stronger binding of USF-2 and Pol II to the promoter of hAT1R gene from the kidney chromatin of TG mice containing haplotype-I as compared to haplotype-II. Fig. 6A and 6B show the PCR amplification of immuno-precipitated DNA from the two haplotypes in the presence of antibodies against USF-2, Pol II and IgG (mock control). Lane 'Non specific primers' shows amplification using non specific primers from a region not containing the respective binding site. Fig. 6C and 6D show the relative band intensities for both the haplotypes for USF-2 and Pol II ChIP assays that were normalized to the band intensities from the input DNA. Error bars are SEM (n=4). *p<0.05 vs. Haplotype-II

Fig. 9: Expression of Pro-inflammatory Markers and Oxidative markers is increased in transgenic mice containing haplotype-I of hAT1R gene: Functional relevance of the up-regulated hAT1R was assessed by relative mRNA expression analysis of the pro-inflammatory genes including IL6, TNFα, CRP and oxidative marker Nox-1 in the kidney of transgenic mice containing haplotype-I (solid bars) and haplotype-II (empty bars) of hAT1R gene using QRT-PCR. Results were normalized to mouse GAPDH. Error bars are SEM (n=6). *p<0.05 vs. Haplotype-II

Fig. 10: Blood Pressure is increased in male transgenic mice containing haplotype-I of hAT1R gene: A: Systolic blood pressure of hAT1R-TG (TG) mice taken over 24 hr by telemetry. Each bar represent hourly mean from 6 male animals taken over 4 days. B: Comparison of 24h average systolic blood pressure of transgenic mice containing either Haplotype-I (solid bars) and Haplotype-II (empty bars). To confirm the role of increased hAT1R in bringing about this difference in SBP, animals were treated with losartan for 24 hr and BP was measured by telemetry. Error bars are SEM (n=6). *p<0.05 vs. Haplotype-II. † indicates p<0.05 vs. Haplotype-I without losartan.
Table I: Analysis of human AT₁R gene polymorphism at -680 in Caucasian subjects

|               | Total Subjects | TT  | GT  | GG  | p=004   |
|---------------|----------------|-----|-----|-----|---------|
| Hypertensive  | 374            | 244 | 114 | 16  |         |
| Normotensive  | 388            | 208 | 159 | 21  |         |

Table II: Sequences of the primers used for the characterization of human AT₁R gene in the hAT₁R transgenic mice (TIS indicates transcription initiation site).

| Primer         | Location     | Sequence                           |
|----------------|--------------|------------------------------------|
| 5’upstream-F   | 43kb 5’TIS   | GAGATGGAGTCTCACTCT                 |
| 5’upstream-R   |              | CATGAGCAGACAATTCTC                 |
| Exon 2-F       | 10kb 3’TIS   | CTCTTAAGATGCAATGTGG                |
| Exon 2-R       |              | CTGCTTCCTCTACTTCT                  |
| Exon 5-F       | 43kb 3’TIS   | TGGCATGGCAGTGAGGT                 |
| Exon 5-R       |              | CAGATGCTCAAGAATGG                  |
| 3’downstream-F | 70kb 3’TIS   | GACACAGTGAAGTGAATG                 |
| 3’downstream-R |              | CTATATGGTTCCACAGGCA                |
Polymorphisms in Haplotype I and II of the hAT,R

-777  -680  -214  -119
HaplI
T    T    A    A

-777  -680  -214  -119
HaplII
A    G    C    G

Fig. 1

Amplified sequence          TGGAT T/G CAAT
Restriction site            GAN  T  C

Fig. 2
Fig. 3

(A) Relative luciferase activity for H295R with Haplotypes I and II.

(B) Relative luciferase activity for A7R5 with Haplotypes I and II.

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Fig. 4

(A) Sequence comparison:
- 214A: C GGG ACC ACG TGA AC
- 214C: C GGG ACC CCG TGA AC
- USF consensus: G TAG GCC ACG TGA CC

(B) Gel electrophoresis showing bands for 214A and 214C with different conditions.
**Generation of TG mice: Structural analysis of the hAT₃R BAC construct**

**(A)**

**(B)**

Fig. 5

Fig. 6
Fig. 7

(A) Relative mRNA expression

(B) Relative mRNA expression

Fig. 8

(A) Gel electrophoresis for USF, Input, IgG, Non-specific primer

(B) Gel electrophoresis for Pol-II, Input, IgG, Non-specific primer

(C) Relative band intensity for Haplotype-I and Haplotype-II

(D) Relative band intensity for Haplotype-I and Haplotype-II
Blood pressure analysis in TG mice containing Haplotype I and II of hAT1R gene

Fig. 9

Fig. 10
A Haplotype of Angiotensin Receptor Associated with Human Hypertension Increases Blood Pressure in Transgenic Mice
Sudhir Jain, Alicia Prater, Varunkumar Pandey, Anita Rana, Nitin Puri and Ashok Kumar

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