The effect of glucocorticoids on regulation of the human angiotensinogen gene and blood pressure

Varunkumar Girijaprasad Pandey

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A Dissertation

entitled

The Effect of Glucocorticoids on Regulation of the Human Angiotensinogen Gene and
Blood Pressure

by

Varunkumar Girijaprasad Pandey

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Doctor of Philosophy Degree in Biomedical Science

_________________________________________
Ashok Kumar PhD, Committee Chair

_________________________________________
Bina Joe PhD, Committee Member

_________________________________________
Andrew Beavis PhD, Committee Member

_________________________________________
Nitin Puri M.D. PhD, Committee Member

_________________________________________
Zahoor Shah PhD, Committee Member

_________________________________________
Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

December 2013
An Abstract of

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Human angiotensinogen gene locus is associated with hypertension. Human angiotensinogen gene (hAGT) has an A/G polymorphism and -6A (rs5051) is associated with increased blood pressure. We have found that hAGT has three additional single nucleotide polymorphisms (SNPs). Variants -1670A, -1562C and -1561T almost always occur with -6A haplotype (Hap -6A); and variants -1670G, -1562G and -1561G almost always occur with -6G haplotype (Hap -6G). We have shown that these polymorphisms affect the binding of glucocorticoid receptor (GR) to the promoter of the hAGT gene. Reporter construct containing 1.8 Kb of the hAGT promoter with Hap -6A has 4 fold increased glucocorticoid induced promoter activity as compared to hap -6G. Therefore, we have generated transgenic mice (TG) containing either Hap -6A or Hap -6G of the hAGT gene to study the regulation of the hAGT gene in an in vivo situation. Since hAGT is not cleaved by mouse rennin, we generated double transgenic mice with human rennin and hAGT gene of either Hap -6A or Hap -6G. We have reported that TG mice Hap -6A of the hAGT gene has increased plasma Angiotensinogen levels and significantly elevated blood pressure as compared to Hap -6G. We hypothesize that these three
additional SNPs in the promoter of the Hap -6A may be predisposing to hypertension in response to increased glucocorticoid levels.

TG mice with hap -6A and Hap -6G were treated with dexamethasone (DEX) (10μg/day in drinking water) for 72 hours. Our Q-RTPCR results show that mice with Hap -6A have significant increase in the mRNA expression of the hAGT in the liver and the kidney on treatment with DEX whereas no significant changes were observed in Hap -6G. Q-RTPCR results were confirmed by western blots showing significant increase in the DEX induced expression of the hAGT protein in the liver and the kidney of the TG mice with Hap -6A. DEX treatment also increased the plasma hAGT protein and Ang-II levels in TG mice with Hap -6A whereas no significant changes were observed in TG mice with Hap -6G. The systolic blood pressure (SBP) of TG mice containing Hap -6A increased by 12mmHg on treatment with DEX as compared to 5mmHg in the mice with Hap -6G. We subjected these mice to increased dosage of DEX at 50μg/day and observed a dose dependent modulation of the SBP. DEX dependent increase in the systolic blood pressure was reduced by treatment with angiotensin receptor blocker losartan.
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# Table of Contents

Abstract .................................................................................................................................................. iii

Acknowledgements .................................................................................................................................. v

Table of Contents .................................................................................................................................... vi

List of Figures ......................................................................................................................................... ix

List of Schematics ...................................................................................................................................... xi

List of Abbreviations ............................................................................................................................... xii

1 Introduction........................................................................................................................................... 1

2 Review of the literature ....................................................................................................................... 4
    2.1 Renin-Angiotensin-Aldosterone System (RAAS) ........................................................................ 4
    2.2 RAAS in Mammalian Physiology ............................................................................................... 7
    2.3 Systemic and Tissue Distribution of RAAS .............................................................................. 15
    2.4 Genetics of Hypertension .......................................................................................................... 17
    2.5 Role of Genetic Variants of the RAAS in Hypertension ............................................................ 18
    2.6 Role of Glucocorticoids in Hypertension .................................................................................... 21
    2.7 The Human Angiotensinogen Gene ............................................................................................ 22
    2.8 Characteristics of the hAGT Protein ............................................................................................ 23
    2.9 Regulation of the hAGT Gene ...................................................................................................... 23
    2.10 Association of the hAGT Gene with Hypertension ................................................................... 24
2.11  Structure of Haplotype -6A and Haplotype -6G of the hAGT Gene……28

2.12  Structure of the hAGT Promoter…………………………………………………29

2.13  Glucocorticoid Response Elements in the hAGT Gene……………….30

2.14  Comparison of the Putative ‘cis’ Elements in the hAGT Gene with
Consensus GRE and HNF-1 Binding Sites……………………………………31

2.15  In vitro Analysis of the Effect of DEX on the Promoter Activity of
Haplotype -6A and Haplotype -6G of the hAGT Gene…………………32

2.16  Generation of Transgenic Mice with Haplotype -6A and Haplotype -6G of
the hAGT Gene ……………………………………………………………33

3  Hypothesis and Specific Aims of the Research…………………………41

4  Materials and Methods…………………………………………………………44

4.1  Animal Care and Use…………………………………………………………44

4.2  Transgenic Mice………………………………………………………………44

4.3  Generation of the Double Transgenic Mice Containing Human AGT and
Human Renin……………………………………………………………………46

4.4  Dexamethasone Treatment………………………………………………..47

4.5  Quantitative Real-Time RT-PCR…………………………………………48

4.6  Western Blot Analysis…………………………………………………………49

4.7  In-vivo Chromatin Immune-Precipitation Assay…………………………49

4.8  Measurement of Plasma Angiotensin-II………………………………51

4.9  Blood Pressure Measurement in Mice……………………………………51

4.10 Statistical Analysis……………………………………………………………53

5  Results………………………………………………………………………………54
5.1 Dexamethasone Induces Expression of the hAGT Gene in the Liver of Transgenic Mice Containing Hap-6A........................................54
5.2 Kidney Shows Increased Expression of the hAGT Gene in Transgenic Mice with Hap-6A in Response to Dexamethasone Treatment.........57
5.3 Treatment with Dexamethasone Causes an Increase in both hAGT as well as Endogenous Mouse AGT in the Adrenals of Transgenic Mice with Haplotype-6A.................................................................60
5.4 ChIP Analysis Shows Binding of GR to the Promoter of the hAGT gene is Increased in the Transgenic Mice with Haplotype -6A on Treatment with Dexamethasone.................................................................63
5.5 DEX Treatment Elicits a Significant Increase in the Plasma hAGT and Ang-II Levels of Transgenic Mice with Haplotype -6A...............68
5.6 Dexamethasone Dependent Modulation of the Systolic Blood Pressure is Affected by the Nucleotide Differences in the ‘cis’ Elements of Hap-6A and Hap-6G of the hAGT Gene.........................................................72

6 Discussion...........................................................................................................75

7 Future Prospects..................................................................................................88

References..............................................................................................................90
List of Figures

2-1 Structure of the hAGT promoter showing transcription factor binding sites from -1714 to -1530 nucleotide sequence .................................................................29
2-2 In vitro analysis of the effect of DEX on the promoter of Hap -6A and Hap -6G of the hAGT gene ............................................................................................................32
2-3 Expression of the hAGT mRNA and hAGT protein levels in the liver and the kidney of the transgenic mice ................................................................................34
2-4 Chromatin Immuno-precipitation assay showing binding of GR, HNF-1α and RNA polymerase to the promoter of the hAGT gene in TG mice .........................36
2-5 Plasma hAGT protein levels in TG mice with Hap -6A and Hap -6G .................37
2-6 Systolic blood pressure of TG mice with Hap -6A and Hap -6G .........................39
2-7 24 hour SBP showing diurnal rhythm in TG mice Hap -6A and Hap -6G ......40
5-1 Effect of dexamethasone on mRNA levels of the hAGT and the mAGT gene in the liver of the TG mice with Hap -6A and Hap -6G ..................................................53
5-2 Effect of dexamethasone on the hAGT protein in the liver of TG mice ..........54
5-3 Effect of dexamethasone on mRNA levels of the hAGT and the mAGT gene in the kidney of the TG mice with Hap -6A and Hap -6G ............................................56
5-4 Effect of dexamethasone on the hAGT protein in the liver of TG mice............57
5-5 Effect of dexamethasone on mRNA levels of the hAGT and the mAGT gene in the adrenals of the TG mice with Hap -6A and Hap -6G ........................................69
5-6 Effect of dexamethasone on mRNA levels of the Cyp11B1 and the Cyp11B2 gene in the adrenals of the TG mice with Hap -6A and Hap -6G .........................................70
5-7 ChIP assay shows binding of GR at the -217 and -1561/-1562 site in the liver of the TG mice with Hap -6A and Hap -6G.................................................................72
5-8 Box-whisker plot showing ChIP data for binding of GR at the -217 and -1561/-1562 site in the liver of the TG mice with Hap -6A and Hap -6G .........................73
5-9 ChIP assay shows binding of GR at the -217 and -1561/-1562 site in the kidney of the TG mice with Hap -6A and Hap -6G.................................................................74
5-10 Box-whisker plot showing ChIP data for binding of GR at the -217 and -1561/-1562 site in the liver of the TG mice with Hap -6A and Hap -6G .........................75
5-11 Effect of dexamethasone on hAGT protein levels in the plasma of the TG mice with Hap -6A and Hap -6G.................................................................77
5-12 Effect of dexamethasone on mRNA levels of the human and the mouse renin gene in the kidneys of the TG mice with Hap -6A and Hap -6G .........................78
5-13 Effect of dexamethasone on Angiotensin-II levels in the plasma of the TG mice with Hap -6A and Hap -6G.................................................................79
5-14 Effect of dexamethasone on the systolic blood pressure (SBP) of the TG mice with Hap -6A and Hap -6G.................................................................81
List of Schematics

2-1  Structure of the hAGT promoter showing transcription factor binding sites from -1714 to -1530 nucleotide sequence .................................................................29

2-2  In vitro analysis of the effect of DEX on the promoter of Hap -6A and Hap -6G of the hAGT gene ...........................................................................................................32

2-3  Expression of the hAGT mRNA and hAGT protein levels in the liver and the kidney of the transgenic mice .................................................................................34

2-4  Chromatin Immuno-precipitation assay showing binding of GR, HNF-1α and RNA polymerase to the promoter of the hAGT gene in TG mice ........................36

2-5  Plasma hAGT protein levels in TG mice with Hap -6A and Hap -6G .................37

2-6  Systolic blood pressure of TG mice with Hap -6A and Hap -6G .........................39

2-7  24 hour SBP showing diurnal rhythm in TG mice Hap -6A and Hap -6G ............40

5-1  Effect of dexamethasone on mRNA levels of the hAGT and the mAGT gene in the liver of the TG mice with Hap -6A and Hap -6G ................................................53

5-2  Effect of dexamethasone on the hAGT protein in the liver of TG mice ............54

5-3  Effect of dexamethasone on mRNA levels of the hAGT and the mAGT gene in the kidney of the TG mice with Hap -6A and Hap -6G ........................................56

5-4  Effect of dexamethasone on the hAGT protein in the liver of TG mice ............57
List of Abbreviations

ACTH ................................................................. Adrenocorticotropic hormone
AGT ................................................................. Angiotensinogen
AMP ................................................................. Adenosine monophosphate
ATP ................................................................. Adenosine triphosphate
ANOVA ............................................................. Analysis of variance
ANG-I ................................................................. Angiotensin-I
ANG-II .............................................................. Angiotensin-II
AGT ................................................................. Angiotensinogen
AT₁R ................................................................. Angiotensin receptor subtype-1
AT₂R ................................................................. Angiotensin receptor subtype-2
ACE ................................................................. Angiotensin converting enzyme
BAC ................................................................. Bacterial artificial chromosome
ChIP ................................................................. Chromatin immune-precipitation
CNS ................................................................. Central nervous system
GFR ................................................................. Glomerular filtration rate
GR ................................................................. Glucocorticoid receptor
GRE ................................................................. Glucocorticoid response element
GWAS ............................................................... Genome wide association study
DEX ................................................................. Dexamethasone
Hap ................................................................. Haplotype
hAGT ............................................................... Human angiotensinogen
HNF ................................................................. Hepatocyte nuclear factor
HPA ................................................................. Hypothalamus pituitary adrenal axis
hRen ................................................................. Human renin
IgG ................................................................. Immunoglobulin G
IP₃ ................................................................. 1,4,5-Inositol phosphate
JAK ................................................................. Janus kinase
JGA ................................................................. Juxta-glomerular apparatus
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mAGT</td>
<td>Mouse angiotensinogen</td>
</tr>
<tr>
<td>mRen</td>
<td>Mouse renin</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phospho-enolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
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<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>Q-RTPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
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<td>TG</td>
<td>Transgenic</td>
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Chapter 1

Introduction

Hypertension affects nearly one third of the adult population in the United States of America. Centers for Disease Control estimates approximately 68 million Americans have hypertension and nearly one third of this population remains undiagnosed. JD Cohen estimated the total economic burden of hypertension related medical expenses to be at $73.4 billion(1). Hypertension increases the risk for cardiovascular, renal and cerebrovascular disease. Clinically hypertension is defined as systolic blood pressure over 140mmHg or diastolic over 90mmHg or both. There are several drugs in clinical use to alleviate symptoms of hypertension including AT1R blockers, β-blockers, ACE inhibitors and diuretics. However, many patients require multiple drugs, at higher dosages, to manage their conditions. At this point, these patients may have already sustained organ damage. Ninety percent of the cases of hypertension have idiopathic etiology and are defined as having essential hypertension. The American Heart Association has reported that hypertension has become increasingly resistant to anti-hypertensive drugs and estimated that 20-30% hypertensive patients could not control their blood pressure with three or more drugs, even when taking them exactly as prescribed (2;3). The understanding of genetic underpinnings and patho-physiology of
hypertension is essential to establish preventive measures and fortify the clinical arsenal against high blood pressure.

Blood pressure is a complex physiological parameter dynamically regulated by robust homeostatic mechanisms. Blood pressure is affected by age, body weight, stress, diet and ancestry. With a gain of every 5% body weight, the chance of hypertension increases by 20-30%. There are a plethora of physiological factors that regulate blood pressure including humoral mediators, vascular reactivity, extracellular fluid volume, vascular caliber, blood viscosity, cardiac output, renal function, blood vessel elasticity, and neural stimulation. Aberrations in the genes that regulate these physiological factors may incur genetic susceptibility to hypertension. Human angiotensinogen gene (hAGT) locus is associated with hypertension. The hAGT gene has -6A/G polymorphism and -6A (rs5051) allele is associated with increased blood pressure (4). We have found that hAGT gene has three additional SNPs. Variants -1670A (rs2493136), -1562C (rs2493135), and -1561T (rs11568016) almost always occur with -6A haplotype (Hap -6A); and variants -1670G, -1562G, and -1561G almost always occur with -6G haplotype (Hap -6G). Our lab has also reported that there is a SNP at -217 position in the hAGT promoter and -217A (rs5049) causes higher plasma hAGT levels and increased blood pressure. We have shown that these polymorphisms affect the binding of glucocorticoid receptor to the promoter. Reporter construct containing 1.8 Kb of the hAGT gene promoter with Hap -6A has 4 fold increased glucocorticoid induced promoter activity as compared to Hap -6G. To examine the effect of these polymorphisms in an in vivo situation, our lab has generated transgenic (TG) mice containing either Hap -6A or Hap -6G of the hAGT gene. Previously our lab has shown that TG mice with Hap -6A of hAGT gene has increased
plasma angiotensinogen levels and significantly elevated blood pressure as compared to the Hap -6G. Since these three additional SNPs affect the glucocorticoid induced promoter activity, we hypothesized that the Hap -6A of the hAGT gene may be a predisposing factor for hypertension in response to increased glucocorticoid levels.

Three month old male double TG mice containing either Hap -6A or Hap -6G along with the human renin gene were treated with dexamethasone (DEX) (2.5µg/mL in drinking water) for 72 hours. Our Q-RTPCR results show that mice with Hap -6A has 2 and 2.06 fold DEX induced mRNA expression of the hAGT gene in the liver and the kidney respectively whereas no significant changes were observed in Hap -6G. Q-RTPCR results were confirmed by western blots showing 1.80, 1.94 and 1.40 fold induction of hAGT protein in liver, kidney and plasma respectively in TG mice with Hap -6A. Using chromatin immuno-precipitation (ChIP) analysis we have shown that DEX treatment increases the binding of glucocorticoid receptors (GR) to the promoter of the hAGT gene in both liver and kidney of the TG mice with Hap -6A of the hAGT gene. The binding of GR to the promoter of the hAGT gene TG mice with Hap -6G was unaffected by DEX administration. DEX treatment also increased the systolic blood pressure (SBP) of TG mice containing Hap -6A by 12 mmHg as compared to 5 mmHg in the mice with Hap -6G. We subjected these TG mice to variable doses of DEX and observed a dose dependent modulation of SBP. DEX dependent increase in the SBP was reduced by losartan.
Chapter 2

Review of Literature

2.1. Renin-Angiotensinogen-Aldosterone System (RAAS)

The renin-angiotensin (RAAS) system is a peptidergic endocrine system central to the regulation of the mean arterial pressure and effective circulating volume. Much like

Schematic.2-1. Structure of juxta-glomerular apparatus (JGA)
other endocrine homeostatic regulators, RAAS operates on a classical negative feedback loop where higher than normal mean arterial pressure or effective circulating volume suppresses the system. The RAAS system plays a critical role in sodium and water homeostasis and maintenance of vascular tone (5). Activation of the renin-angiotensin aldosterone system (RAAS) occurs primarily as a compensatory mechanism to restore blood volume and maintain kidney perfusion by retaining salt and water.

Schematic 2-2. Renin-Angiotensin-Aldosterone System (RAAS)

Classical teachings of the RAAS describe renin production as the rate-limiting step in the regulation of RAAS. Renin is synthesized (Schematic 1) in specialized renal tissues—the juxta-glomerular apparatus (JGA). As the name suggests, JGA is formed when the thick ascending limb (TAL) loops back in to the renal cortex and lies adjacent
to the glomerulus (6). Evolution of this design brings a distal segment of the nephron in close proximity to the vasculature traversing the glomerulus. This allows for cross talk between the TAL and the glomerular arterioles, where physical composition of the urine affects glomerular arterioles and the glomerular filtration rate (GFR). Renin is synthesized and stored in an inactive form called pro-renin in the juxtaglomerular cells (JG cells) of the JGA. The JG cells are modified smooth muscle cells located in the walls of the afferent arterioles immediately proximal to the glomeruli. Most of the renin enters the renal blood and then passes out of the kidneys to circulate throughout the entire body. However, small amounts of the renin do remain in the local fluids of the kidney and initiate several intra-renal functions.

Schematic.2-3. Formation of angiotensin polypeptides

Angiotensinogen (AGT) is an α-2-globulin that is produced constitutively and released into the circulation mainly by the liver. AGT is a 485 amino acid protein cleaved by
circulating renin to yield the decapeptide angiotensin-I (Ang-I). Ang-I is subsequently cleaved by angiotensin converting enzyme (ACE) to the principal biologically active mediator of the RAAS system angiotensin II (Ang-II). ANG-II in turn promotes aldosterone release from the adrenal cortex (Schematic 2-2, 2-3).

Apart from the central pathway leading from AGT to ANG-II, smaller quantities of other peptides are also formed in the RAAS. Unlike ACE, which cleaves two carboxyl-amino acids, ACE2 is a mono-carboxypeptidase. Ang-II is a substrate for ACE2 and undergoes cleavage to yield Ang1-7. Similarly, Ang1-7 can be formed via sequential processing of Ang-I by ACE2 and ACE (7). Tissue aminopeptidases (AP) can metabolize Ang-II to the recently discovered Ang2-8 also referred to as Ang-IV (8) (Schematic 1-3).

2.2. RAAS in Mammalian Physiology

Renin is an aspartyl protease that is synthesized as prorenin, a pro-enzyme that is converted into renin by cleavage of a 43-amino-acid segment from the N-terminal end. This activation process occurs exclusively in the JGA of the kidney and is followed by the release of renin into the circulation system. Although synthesized in only a few tissues (eyes, adrenal glands, testes, ovaries, and brain), prorenin represents between 70% and 90% of the total plasma renin. The local actions of renin are thus mediated by kidney-derived renin that is released into the circulation system and taken up by tissues. Renin receptors have been localized in glomerular mesangium and vascular smooth muscle cells within the sub-endothelium of glomerular and coronary arteries. Cells
transfected with receptor cDNA resulted in the expression of a membrane protein that specifically binds renin and prorenin with high affinity. Labeling studies have demonstrated high-affinity binding (Kd = 0.4nM) of renin to receptors on cultured human mesangial cells (9).

The binding of renin to its receptor has multiple and far-reaching consequences. Receptor binding induces a 4-fold increase in the catalytic conversion of angiotensinogen to Ang-I, suggesting that the cell surface is an important site of Ang-I generation. Once bound, renin triggers a series of intracellular events that culminate in activation of the mitogen-activated protein kinases ERK1 (p44) and ERK2 (p42), which are involved in cell hypertrophy and proliferation (10). At physiologic levels, renin enhances the incorporation of 3H-thymidine into cells, with no increase in cell numbers; increases transforming growth factor beta in mesangial cells (suggesting up regulation through a receptor-mediated mechanism, independent of Ang-II generation or action) (11); and activates the synthesis of plasminogen activator-1 and fibrotic extracellular components such as fibronectin and collagen (suggesting that renin may contribute to fibrotic disease). Over-expression of the renin receptor in arterial smooth muscle cells of transgenic rats resulted in high BP levels, increased heart rate, and significant elevation of plasma aldosterone levels, effects that were attributed to local activation of the intra-adrenal RAAS (12).

When bound to the renin receptor, the catalytic activity of prorenin is increased and parallels that of renin. Elevated prorenin levels have been correlated with microvascular complications of diabetes, and lesions mimicking diabetic nephrosclerosis are present in transgenic rats expressing prorenin. Rats with streptozotocin-induced diabetes
have high levels of prorenin, Ang I, and Ang II, but not of renin and ACE. Non-proteolytic activation of prorenin to its enzymatically active state occurs through the binding of certain carbohydrate substances or the renin receptor. When non-proteolytic conversion of prorenin is inhibited with a blocking peptide, levels of renal Ang I and Ang II are reduced and the development of nephropathy is attenuated, thus substantiating that prorenin has a role in the progression of diabetic renal damage (13). Thus, elevated levels of prorenin, synthesized locally in the vasculature, cardiac cells or the kidneys could potentially lead to elevated Ang-I & II levels at these sites with consequent effects expected of activated RAAS.

Ang-II is the primary bioactive molecule of the RAAS. Ang-II couples to GPCRs, angiotensin receptors AT1R and AT2R. Most of the known biological effects of Ang-II are mediated by the AT1R receptor. Activation of AT2R receptor counterbalances many of the effects of the AT1R receptors by having antiproliferative, proapoptotic, vasodilatory, natriuretic, and antihypertensive effects (14-20). The AT2R receptor is distributed widely in fetal tissues, but its distribution is more restricted in adults. Although the AT2R receptor generally is conceptualized as a cardiovascular protective receptor, its overexpression and activation may contribute to myocyte hypertrophy and cardiac fibrosis. Expression of AT2R receptors is up regulated in cardiovascular diseases, including heart failure, cardiac fibrosis, and ischemic heart disease; however, the significance of increased AT2R receptor expression is unclear. The Mas receptor mediates the effects of Ang(1–7), which include vasodilation and anti-proliferation. Deletion of the Mas gene in transgenic mice reveals cardiac dysfunction (21-23).
AT\textsubscript{1}R receptors couple to several heterotrimeric G proteins, including Gq, G12/13, and Gi. In most cell types, AT\textsubscript{1}R receptors couple to Gq to activate the PLC–IP\textsubscript{3}–Ca\textsuperscript{2+} pathway. Secondary to Gq activation, activation of PKC, PLA\textsubscript{2}, and PLD and eicosanoid production, as well as activation of Ca\textsuperscript{2+}-dependent and MAP kinases and the Ca\textsuperscript{2+}–calmodulin–dependent activation of nitric oxide synthase (NOS) can occur (24). Activation of Gi may occur and will reduce the activity of adenylyl cyclase, lowering cellular cyclic AMP content. The subunits of Gi and activation of G12/13 leads to activation of tyrosine kinases and small G proteins such as Rho. Ultimately, the JAK/STAT pathway may be activated and a variety of transcriptional regulatory factors induced. By these mechanisms, angiotensin influences the expression of a host of gene products relating to cell growth and the production of components of the extracellular matrix. AT\textsubscript{1}R also stimulates the activity of a membrane-bound NADH/NADPH oxidase that generates reactive oxygen species (ROS) (25). ROS may contribute to biochemical effects (activation of MAP kinase, tyrosine kinase, and phosphatases; inactivation of NO; and expression of monocyte chemo-attractant protein-1) and physiological effects (acute effects on renal function, chronic effects on blood pressure, and vascular hypertrophy and inflammation).

Ang-II is one of the central regulators of cardiovascular and renal function. It has a rapid pressor, a slow pressor and a long term remodeling effect on these systems. RAAS, primarily via effects of Ang-II, regulates Na balance, the effective circulating volume and the mean arterial pressure (26). Modest increase in plasma concentrations of Ang-II acutely raises blood pressure; on a molar basis, Ang-II is \sim 40 times more potent than norepinephrine (NE); the EC\textsubscript{50} of ANG-II for acutely raising arterial blood pressure

10
is ~0.3nM. This pressor response to Ang-II is due to a swift increase in total peripheral resistance—a response that helps to maintain arterial blood pressure in the face of an acute hypotensive challenge (e.g., blood loss or vasodilation). Although Ang-II increases cardiac contractility directly (via opening voltage-gated Ca2+ channels in cardiac myocytes) and increases heart rate indirectly (via facilitation of sympathetic tone, enhanced adrenergic neurotransmission, and adrenal catecholamine release), the rapid increase in arterial blood pressure activates a baroreceptor reflex that decreases sympathetic tone and increases vagal tone. Thus, depending on the physiological state, Ang-II may increase, decrease, or not change cardiac contractility, heart rate, and cardiac output. The long-term effects of Ang-II are more complex. The slow pressor effect of Ang-II primarily relies upon the regulation of the effective circulating volume; this is achieved in the kidney. Very low concentrations of Ang-II stimulate Na+/H+ exchange in the proximal tubule—an effect that increases Na+, Cl–, and bicarbonate reabsorption. Approximately 20-30% of the bicarbonate handled by the nephron may be affected by this mechanism. Ang-II also increases the expression of the Na+–glucose symporter in the proximal tubule. Paradoxically, at high concentrations, Ang-II may inhibit Na+ transport in the proximal tubule. Ang-II also directly stimulates the Na+–K+–2Cl– symporter in the thick ascending limb. The proximal tubule secretes angiotensinogen, and the connecting tubule releases renin, so a paracrine tubular RAAS may contribute to Na+ reabsorption.

Ang-II stimulates the zona glomerulosa of the adrenal cortex to increase the synthesis and secretion of aldosterone, and augments responses to other stimuli (e.g., ACTH, K+) (27). Increased output of aldosterone is elicited by concentrations of Ang-II
that have little or no acute effect on blood pressure. Aldosterone acts on the distal and collecting tubules to cause retention of Na\(^+\) and excretion of K\(^+\) and H\(^+\) (28). The stimulant effect of Ang-II on aldosterone synthesis and release is enhanced under conditions of hyponatremia or hyperkalemia and is reduced when concentrations of Na\(^+\) and K\(^+\) in plasma are altered in the opposite directions. Additionally, Ang-II regulates Na balance via its effect on renal hemodynamics. Ang-II reduces renal blood flow and renal excretory function by directly constricting the renal vascular smooth muscle, by enhancing renal sympathetic tone (a CNS effect), and by facilitating renal adrenergic transmission (an intrarenal effect). Ang-II influences glomerular filtration rate (GFR) by several mechanisms: constriction of the afferent arterioles reduces intraglomerular pressure and tends to reduce GFR; also, contraction of mesangial cells, which decreases the capillary surface area within the glomerulus available for filtration and also tends to decrease GFR. However, constriction of efferent arterioles increases intraglomerular pressure and tends to increase GFR. Whatsoever the effect on GFR, Ang-II-induced constriction of either of the arterioles will reduce renal plasma flow and thus increasing the filtration fraction (GFR/RPF). This will increase oncotic pressure in the peritubular capillaries and facilitate Na-reabsorption by Starling’s forces. Normally, GFR is slightly reduced by Ang-II; however, during renal artery hypotension, the effects of Ang-II on the efferent arteriole predominate so that Ang-II increases GFR. Thus, blockade of the RAAS may cause acute renal failure in patients with bilateral renal artery stenosis or in patients with unilateral stenosis who have only a single kidney (Scheme 4 summarizes the effects of Ang-II playing a role in the regulation of MAP)

12
Schematic.2-4. Effects of Ang-II on cardiovascular functions.

SNS: sympathetic nervous stimulation, NE: norepinephrine, CNS; central nervous system, AM: adrenal medulla, NHE: Na\(^+\)/H\(^+\) exchange activity, PT: proximal tubules, EA: efferent arterioles, TPR: Total peripheral resistance

Systemic RAAS operates on the classical negative feedback loop. This is achieved at the level of renin secretion. The first mechanism is the macula densa pathway. The macula densa lies adjacent to the juxtaglomerular cells and is composed of specialized columnar epithelial cells in the wall of that portion of the cortical thick ascending limb that passes between the afferent and efferent arterioles of the glomerulus. A change in NaCl reabsorption by the macula densa results in the transmission to nearby juxtaglomerular cells of chemical signals that modify renin release. Increases in NaCl flux across the macula densa inhibit renin release, whereas
decreases in NaCl flux stimulate renin release. ATP, adenosine, and prostaglandins modulate the macula densa pathway. The second mechanism controlling renin release is the intrarenal baroreceptor pathway. Increases and decreases in blood pressure or renal perfusion pressure in the preglomerular vessels inhibit and stimulate renin release, respectively. The immediate stimulus to secretion is believed to be reduced tension within the wall of the afferent arteriole. The release of renal prostaglandins and biomechanical coupling via stretch-activated ion channels may mediate in part the intrarenal baroreceptor pathway (Wang et al., 1999). Finally, the third mechanism, the adrenergic receptor pathway is mediated by the release of norepinephrine from postganglionic sympathetic nerves; activation of β1 adrenergic receptors on juxtaglomerular cells enhances renin secretion. The three mechanisms regulating renin release are embedded in a feedback regulation (Schematic 2-5).

Increased renin secretion enhances the formation of Ang-II, which stimulates AT$_1$R receptors on juxtaglomerular cells to inhibit renin release. Ang-II increases arterial blood pressure via AT$_1$R receptors; this effect inhibits renin release by: activating high-pressure baroreceptors, thereby reducing renal sympathetic tone; increasing pressure in the preglomerular vessels and reducing NaCl reabsorption in the proximal tubule (pressure natriuresis), which increases tubular delivery of NaCl to the macula densa.
2.3. Systemic and Tissue Distribution of RAAS

The hAGT molecule is primarily synthesized in the liver and secreted in plasma. However, organ specific expression of hAGT is well documented in various tissues including adipose, kidney, heart, vasculature, adrenals and brain. Drugs that block the RAAS are known to alleviate high blood pressure in patients even with low circulating RAAS components implying the significance of local RAAS system(29). An active local RAAS system in a tissue is characterized by expression of all the components of RAAS.
essential for de novo generation of vaso-active octa-peptide angiotensin-II. Adipocytes express high levels of AGT and are second major contributors to the systemic angiotensinogen. Studies have shown increased plasma AGT levels and blood pressure in transgenic mice with adipocyte specific over-expression of AGT (30;31). These studies suggest that the adipocyte derived AGT in obese human subjects may contribute to increased systemic AGT levels and increased blood pressure. Kidney has an active local RAAS system wherein renin is expressed in the juxtaglomerular cells and AGT is synthesized in the proximal tubular cells(32). Both renin and AGT expression are affected by the plasma Ang-II levels. Increased Ang-II levels alter the pressure natriuresis curve and thereby increase blood pressure. Dysregulation of RAAS components in the kidney is well documented in relation to high blood pressure, cardiovascular and renal diseases. However, the role of genetic variants of angiotensinogen and its effects on local and systemic RAAS are yet to be understood. Glial cells throughout the brain express AGT. Astrocytes and neurons present in nuclei of the brain that control cardiovascular functions express abundant levels of AGT (33;34). Blood pressure is modestly elevated by over expression of AGT in both glial and neuronal cells(35) Angiotensin is also implicated to function as a neurotransmitter in regulating the central autonomic nervous system(36) Transgenic mice with brain specific activation of RAAS have shown increased fluid turnover, elevated sympathetic nervous activity and increased metabolic rate(37). Local AGT synthesized in the brain has been implicated in high blood pressure; the regulators and factors of dysregulation remain elusive. Adrenal glands play a critical role in Na-water homeostasis via the mineralocorticoid steroid aldosterone. Circulating as well as tissue generated Ang-II functions as the principal agonist for synthesis of


aldosterone(38). The intricate interplay between the Ang-II and aldosterone has a profound effect on maintenance of extracellular fluid volume. Ang-II generated by the cardiac RAAS has been implicated in cardiac tissue remodeling and hypertrophy by modulation of JAK-STAT pathway (39). Ang-II synthesized by active RAAS components in the endothelium modulates the vascular tone and leads to alterations in blood pressure. Locally produced Ang-II in the vasculature also triggers the synthesis of vasoconstrictors including prostaglandins and endothelins as well as inhibit the production of vasodilatory endothelial nitric oxide. Overall, systemic and local expression of AGT has significant implications in regulation and dys-regulation of the blood pressure and metabolic phenotype. The factors that alter the expression of AGT in local tissues and/or at the systemic levels may lead to differential physiological outcomes necessitating a detailed investigation of regulation of the human AGT gene.

2.4. Genetics of Hypertension

Multiple strategies including association studies from well-defined patient cohorts, animal models, candidate gene approach and genome wide association studies (GWAS) have been utilized to determine the genetic causes of hypertension; with only a modest success. Hypertension is a polygenic disorder with up to 60% heritability as estimated by twin genetic studies (40;41). However, less than 3% of this genetic inheritance is attributable to loci discovered by the existing genetic study designs including genome wide association studies (GWAS)(42). The hypertensive phenotype presents unique challenges to the geneticists because of the intricate nature of interactions
between various physiological pathways that regulate blood pressure including extracellular fluid volume homeostasis, cardiac contractility, and vascular tone through renal, endocrine and neural mechanisms. This missing heritability could partially be explained by interactions of genes with the environment, including salt intake, body weight, gender, and exposure to stress. Several candidate genes from diverse pathways have been associated with hypertension. However, molecular mechanisms contributing to the phenotype have largely remained elusive. One of the key regulatory pathways that profoundly affect blood pressure is the Renin-Angiotensin-Aldosterone (RAAS) system. Consequently, genetic perturbations in each component of the RAAS system have been extensively investigated for their role in predisposition for hypertension.

2.5. Role of Genetic Variants of the RAAS in Hypertension

Single nucleotide polymorphisms (SNPs) are single nucleotide variations present in the DNA sequences in the genome. These alterations occur at a frequency of more than one per thousand nucleotides and allow for possible introduction of new alleles. The distribution of these SNPs in the genome is heterogeneous in nature with frequent occurrences in the non-coding vs. the coding regions. The effect of these SNPs on the phenotype depends on the location of their occurrence. SNP in the coding region can result in changes in the protein structure and/or function, if the amino acid is altered by non-synonymous codon modifications. Introduction of a stop codon by SNP can also result in loss of function of a protein. However, alterations in the coding regions of the genes caused by SNPs that deleteriously affect the phenotype are much less frequent and
seldom inherited. SNPs in the regulatory non coding regions (‘cis’ elements) of the gene with minor to moderate effect size on the phenotypes are common and transmitted to subsequent generations. Humans have millions of these SNP variants throughout the genome. Genome wide association studies (GWAS) analyzed millions of these SNPs using state-of-the-art gene chip technologies to detect associated SNPs in complex diseases across several hundred thousands of human subjects. Solitary or several SNP/s affecting a specific pathway with variable quantum effect sizes have a cumulative influence on the phenotype and in some cases incur predisposition to certain disease/s. The effect of SNP/s on a susceptible phenotype/s depends on the interaction of genome with the transcription factor (TF) milieu in various cell types. Consequently, SNPs in the ‘cis’ element of the gene interacts with various physiological and environmental stimuli via TFs to modulate gene expression. Many of these SNPs have been implicated in complex diseases including hypertension, diabetes and metabolic syndrome. As hypothesized by Pickering (43), essential hypertension is characterized as a polygenic disorder wherein cumulative burden of multiple susceptibility factors manifests as the disease phenotype. The investigation of these susceptibility factors (SNPs in most cases) is essential to understand the etiology of hypertension as well as to design preventive and therapeutic measures. Genetic linkage studies on hypertensive patients have discovered several SNPs distributed across all the components of RAAS system. Fuchs et al showed two SNPs with functional role in the human renin gene at -5432 and -5312 by in vitro analysis but could not explain the effect on blood pressure (44). The SNP rs5707 in the homozygous GG genotype was associated with hypertension in post-menopausal women of Spanish ancestry indicating a possible interaction of estrogen with renin gene,
however, the mechanisms remain unclear (45). Angiotensin converting enzyme has an Insertion/Deletion (I/D) genetic variant defined by the presence or absence of a 287-bp fragment spanning intron 16 of the gene that shows association with differential plasma ACE levels without any significant effect on blood pressure. (46). The phenotypes expressing higher levels of ACE exposed to higher levels of angiotensin-I show elevation of blood pressure demonstrating a cumulative effect dependent on gene-gene and gene-environment interaction (22). There are 2 SNPs in the AT₁R gene being extensively scrutinized for hypertensive phenotypes; a SNP that creates a non-synonymous change (T573C) and 1166A/C polymorphism in the 3’-UTR of the gene that alters the interaction with microRNA 155 (miR155) resulting in increased expression (47). The in-vivo significance of AT₁R polymorphisms are yet to be understood. Although, there is a -344 T/C polymorphism in the CYP11B2 gene that synthesizes aldosterone associated with left ventricular hypertrophy, the underlying molecular mechanisms remain elusive (48). There are at least 24 SNPs identified in the hAGT gene. Since increased AGT levels in the plasma is associated with high blood pressure, the study of these polymorphisms is essential to understand the regulation of the hAGT gene and its interaction with gender, BMI and environment.
2.6. Role of Glucocorticoids in Hypertension

Glucocorticoids are steroid hormones produced primarily in the adrenal cortex. The steroid hormones cortisol and corticosterone are major glucocorticoid hormones in the human body and affect physiological mechanisms in nearly all the cell types. Cortisol levels are triggered in response to stress mediated by the hypothalamus-pituitary-adrenal (HPA) axis. Glucocorticoids are also widely used in current medical regimes to alleviate symptoms of allergy, asthma and organ transplantation as well as to compensate for disorders related to adrenal insufficiency. Increased glucocorticoid levels either by physiological mechanisms as in case of Cushing’s syndrome or by pharmacological intervention in cases of allergy, asthma or transplantation causes hypertension (49). Glucocorticoids are known to promiscuously activate mineralocorticoid receptors and modulate kidney function resulting in hypertension (50). Although most of the effect of glucocorticoid treatment on blood pressure is mediated by MR, role of the glucocorticoid receptors remains largely underappreciated. Selective glucocorticoids that do not interact with MR are also known to increase the blood pressure (51). This suggests that genes that are regulated by GR and can affect the vascular tone may be involved in rapid modulation of blood pressure. In this regard, hAGT gene could be a plausible candidate for rapid increase of blood pressure in response to glucocorticoids. Transgenic mice with adipose tissue specific amplification of cortisol shows increased plasma AGT, Ang II and aldosterone levels and develop a hypertensive phenotype (52). To investigate whether
differential regulation of the hAGT gene by glucocorticoids caused hypertension in a subset of population, we focused on the ‘cis’ element of the hAGT gene.

2.7. The Human Angiotensinogen Gene

The hAGT gene belongs to the serpine gene superfamily and remains well conserved among vertebrates. The hAGT gene is a single copy gene located on chromosome one (1q42-q43) and spans about 12.1 kilobases comprising five exons and four introns and encodes a precursor 485 amino acid polypeptide weighing 53kD. The first exon codes for the 5'-untranslated region of the mRNA. The second exon codes for a signal peptide that is 33 residues and the first 252 amino acids (59%) of the mature protein. Mature hAGT contains 452 amino acids; the first ten of them correspond to Angiotensin I generated after cleavage of precursor molecule by renin.
2.8. Characteristics of the hAGT Protein

Human AGT is a globular glycoprotein with a molecular mass between 53 and 61 kDa, depending on its state of glycosylation. The protein contains four putative N-linked glycosylation sites (Asn-X-Ser/Thr) that can be the origins of complex glycosylation chains. Although the role of this glycosylation process is not known in humans, such glycosylation is known to alter the AGT clearance rate in rats. The in vivo half-lives of two differently glycosylated forms of rat AGT are compatible with a two-compartment model and suggest that the more highly glycosylated form is secreted faster by the liver and eliminated more rapidly by the kidney than the less glycosylated form (53). The only known property of hAGT molecule is to act as the substrate for renin. The concentrations of hAGT levels in the plasma are lower than the Michaelis constant ($K_m$) for renin (54). Therefore, increase in the plasma hAGT levels results in increased plasma Ang II concentrations in physiological conditions leading to elevated blood pressure. Redox status of angiotensinogen molecule is also reported to have an impact on AGT-renin reaction and formation of Ang-II (55).

2.9. Regulation of the hAGT Gene

The regulation of the hAGT gene primarily occurs at the transcriptional level. The plasma levels of renin, Ang II and aldosterone have profound effects on the regulation of hAGT gene in different tissues depending on the TF milieu. Glucocorticoids, estrogen and acute phase proteins are known to up regulate expression of the hAGT gene. There are multiple putative cis-acting elements in the 5’ upstream region of the transcription...
initiation site of the hAGT gene that interact with transcription factors including estrogen receptors(ERs), glucocorticoid receptors(GR) and signal transducer and activator of transcription 3(STAT3). SNPs in regulatory regions of the hAGT gene may alter the binding of transcription factors with the ‘cis’ element and consequently affect the transcriptional regulation of the gene. Glucocorticoids are well documented as transcriptional regulators of hAGT gene.

2.10. Association of the hAGT Gene with Hypertension

The hAGT gene is associated with essential hypertension in Caucasians, Asian Indians and Japanese subjects (56-58). Jeunemaître et al showed that there is an SNP rs699 (T/C) at position 704 in exon 2 of the hAGT gene; rs699 is associated with increased plasma AGT concentrations in hypertensive subjects selected from two independent population cohorts derived from Utah and France (59). The SNP rs699 (T→C) causes a methionine to threonine (M→T) amino acid change at position 268 in the hAGT polypeptide sequence commonly known as the variant M235T of the hAGT gene. However, the M235T variation in the amino acid sequence does not alter the kinetics of renin-angiotensinogen reaction as it occurs far from the renin cleavage site suggesting that the functional polymorphism may be lying elsewhere in the hAGT gene. Inoue et al reported a new SNP, rs5051 (G/A) polymorphism at the -6 position in the proximal promoter of the hAGT gene and -6A is strongly associated with hypertension. The variant -6A occurs in complete linkage disequilibrium (LD) with 235T variant and shows higher basal transcriptional activity and hAGT mRNA in hypertensive human
samples carrying -6A allele (60). A number of genetic association and meta-analysis studies have shown homozygous -6A and 235T variant associated with hypertension in multiple population cohorts (61-63). However, a large number of studies have refuted association of the hAGT gene with hypertension (64-66). A large scale gene-centric analysis comprising of 84367 subjects and encompassing approximately 2000 genes implied in cardiovascular regulation identified hAGT gene locus (rs2004776) to be associated with hypertension (67). A few other SNPs in the hAGT gene, including -20C, -217A, -532T and -793A, -1074T were shown to be associated with increased plasma AGT levels and blood pressure depending on the ethnicity and gender (38-40). Although the contribution of individual SNPs on the hAGT gene expression and blood pressure remains unclear, Wu et al reported that haplotypes of the hAGT gene containing several SNPs in LD accounted for approximately 7% of the variance in plasma hAGT levels (41). Scott Watkins et al reported six major haplotypes (H1 to H6) of the hAGT gene based on LD between 24 SNPs spanning across the hAGT gene with frequencies >5% (Schematic.2-7). The H4 haplotype shows -6A allele (rs5051) is in high LD with 1164A (rs2004776) and may be a predisposing factor for essential hypertension (68). Patients with H4 haplotype of the hAGT gene have significantly elevated plasma hAGT levels. The haplotype H4 contains 4 SNPs in the promoter region of the hAGT gene in LD (r^2≥0.85) including -6A, -217A, -532T, 1074T, -1178G that were reported to be associated with elevated plasma hAGT levels. The plasma hAGT levels were negatively associated with the haplotypes H1 and H2 showing the protective nature of these haplotypes. Johnson et al. showed that the polymorphism 1164A (rs2004776) found in the haplotype H4 in the intron1 of the hAGT gene is strongly associated with
hypertension (67). The haplotype -6A in our study is similar to the haplotype H4 shown by Scott Williams et al containing 1164A, -6A,-217A, -532T along with the SNPs in further upstream regions including -1561T, -1562C and -1670A. Therefore, it is plausible that the susceptibility of haplotype H4 to hypertension may be because of the SNPs that are in linkage disequilibrium with rs5051 including three additional SNPs discovered by our research group including -1561T, -1562C and -1670A in the promoter of the hAGT gene.
Schematic 2.7. Haplotypes of the hAGT gene discovered by Scott Watkins et al; Hap H4 has both rs5051 and rs2004776 in linkage disequilibrium. * indicates that Haplotype H1 and H2 are protective against hypertension with $p = 0.029$ and + indicates that Haplotype H4 is associated with hypertension at $p = 0.053$. Haplotype H4 has -6A, -20A and -217A in linkage disequilibrium.
2.11. Structure of Haplotype -6A and Haplotype -6G of the hAGT Gene

Transgenic mice containing complete five exons and four introns along with 1.2 kb of the promoter region with either -6A/235T or -6G/235M variants of the hAGT gene failed to show any difference in the tissue specific expression of hAGT or blood pressure (69). Since, rs5051 does not account for the difference in hAGT expression and blood pressure, we hypothesized that rs5051 may be only a biomarker and the functional polymorphism/s may be lying elsewhere in the hAGT gene. We have found that hAGT gene has three additional SNPs (A/G at -1670, C/G at -1562 and T/G at -1561) and surprisingly variants -1670A, -1562C and -1561T almost always occur with variant -6A (Schematic.2-8). Therefore hAGT gene may be subdivided in either haplotype -6A (containing -6A, -1561T, -1562C, -1670A) or haplotype -6G (containing -6G, -217G, -1561G, -1562G, -1670G).

Schematic.2-8. Description of the haplotype -6A (Hap -6A) and haplotype -6G (Hap -6G) of the hAGT gene
2.12. Structure of the hAGT promoter

**Figure 2-1.** The nucleotide sequence located between -1714 and -1530 of the hAGT gene contains binding sites of multiple transcription factors including hepatocyte nuclear factor-1 (HNF-1), hepatocyte nuclear factor-3 (HNF-3), CCAAT enhancer binding proteins (C/EBP), stimulatory protein (SP-1) and glucocorticoid receptor (GR). The sequence shown in the picture is representative of the haplotype -6G with variant G at position -1561, -1562 and -1670.
2.13. Glucocorticoid Response Elements in the hAGT Gene

There are multiple putative GRE elements in the non-coding regulatory region of the hAGT gene with more than 70% similarity to the consensus GRE sequence. The SNPs at the -217 position alters the binding of GR. Our lab has previously shown that this SNP at the -217 position in the proximal promoter of the hAGT gene affects the basal and glucocorticoid induced promoter activity (70). The SNPs T/G at the -1561 and C/G at the -1562 position of the hAGT gene alters the GR binding sites. The investigation of contribution of other GRE sites in the transcriptional regulation of the hAGT gene remains to be explored.

**Schematic.2-9. GRE binding sites encompassing the hAGT gene.** The numbers after GR represent percentage similarity to consensus GRE and the numbers in brackets represent position of the nucleotide from the transcription initiation site.
2.14. Comparison of the Putative ‘cis’ Elements in the hAGT Gene with Consensus GRE and HNF-1 Binding Sites

Our transfac analysis of the hAGT gene shows that the SNP at -1670 position alters the HNF-1 binding site. The occurrence of nucleotide A at the -1670 position is similar to the consensus HNF-1 sequence and implies stronger interaction with HNF-1 protein. * indicates the -1670 position. Nucleotide sequence of -6A haplotype of the hAGT gene promoter that contains -1561T and -1562C has stronger homology with consensus GRE compared to -1561G and -1562G present in -6G haplotype. * indicates the -1562 position showing C/G polymorphism.

<table>
<thead>
<tr>
<th>G T T A A T G/T A A/T</th>
<th>T N A C</th>
<th>Consensus HNF1</th>
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<td>-1670A, Haplotype -6A</td>
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<table>
<thead>
<tr>
<th>A G N T C N N N N T G T T C T</th>
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<tr>
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<td>-1562C/-1561T Haplotype -6A</td>
</tr>
<tr>
<td>A G G T G G A A T T G T T A C</td>
<td>-1562G/-1561G Haplotype -6G</td>
</tr>
</tbody>
</table>

Schematic.2-10. Nucleotide sequence at the -1670 A/G SNP and -1561/-1562 CT/GG SNPs in the promoter of the hAGT gene
2.15. In vitro Analysis of the Effect of DEX on the Promoter Activity of Hap -6A and Hap -6G of the hAGT Gene

![Bar graph showing relative luciferase activity for HepG2 cells with and without Dex treatment for Hap -6A and Hap -6G.]

**Figure 2-2.** *In vitro* analysis showed that the reporter construct containing 1.8 Kb of the hAGT gene promoter with Hap -6A has 4.7 fold increased glucocorticoid induced promoter activity human liver cells as compared to Hap -6G.

Since previous studies have suggested that -6A allele of hAGT gene is associated with human hypertension and our in vitro studies suggested that Hap -6A of the hAGT gene has increased basal and glucocorticoid induced promoter activity as compared to Hap -6G, we hypothesized that the Hap -6A of the hAGT gene may be a predisposing factor for hypertension in response to increased glucocorticoid levels. Transgenic mice are at present the most rigorous system available for identifying and characterizing cis-acting DNA elements in an in-vivo system. Therefore, we generated transgenic mice.
containing either haplotype -6A or haplotype -6G of the hAGT gene to investigate the effect of glucocorticoids on regulation of the hAGT gene and the blood pressure.

2.16 Generation of Transgenic Mice with Hap -6A and Hap -6G of the hAGT Gene.

Our lab has generated double transgenic mice containing human renin gene and either -6A or -6G haplotype of the hAGT gene to understand the physiological role of these haplotypes in an in vivo situation.

2.16.1. The hAGT mRNA expression is increased in the liver and kidney of transgenic mice containing haplotype -6A of the hAGT gene as compared to the haplotype -6G.

Since liver and kidney are the most important sites for the expression of AGT gene, our research group examined the effect of -6A and -6G haplotypes on hAGT mRNA level in these tissues of transgenic mice by Q-RT-PCR. Quantitation of RNA level in -6A haplotype was performed using RNA level in -6G haplotype as one. Results of these experiments show that AGT mRNA level in the liver of transgenic mice containing -6A haplotype is increased by about 2.5 fold and in the kidney it was increased by 2.1 fold as compared to the transgenic mice containing -6G haplotype (Fig. 3A). A typical Western blot is shown in Fig. 3B and quantitation of AGT protein level normalized to the β-actin is shown in Fig. 3C. The hAGT protein level was increased by about 1.6 fold in transgenic mice with haplotype -6A as compared to haplotype -6G.
Figure 2-3. The hAGT expression is increased in the liver and kidney of double transgenic mice containing -6A haplotype of the hAGT gene as compared to -6G haplotype. (A) RNA from the liver and kidney of transgenic mice containing either -6A or -6G haplotype of the hAGT gene was analyzed by QRT-PCR. (B) Western blot analysis of hAGT in liver of transgenic mice containing -6A or -6G haplotype of the hAGT gene. (C) Quantitation of hAGT protein in livers of transgenic mice containing -6A or -6G haplotype of the hAGT gene as determined by Western blot. Quantitation was performed after normalization with β-actin. *p<0.05 against Hap -6G (71)
2.16.2. Chromatin immunoprecipitation assay showed that HNF-1α, GR, and RNA polymerase have higher affinity to the chromatin of liver from transgenic mice containing -6A haplotype of the hAGT gene as compared to -6G haplotype.

Since our studies described here have shown that: (a) nucleotide sequence of hAGT gene promoter containing -1670A has stronger homology with HNF-1 consensus binding site as compared to -1670G, (b) nucleotide sequence containing -1562C and -1561T has stronger homology with consensus GRE as compared -1562G and -1561G, and (c) previous studies have shown that nucleotide sequence containing -217A has stronger affinity for GR as compared to -217G (72), we wanted to confirm the effect of these polymorphisms on the binding of their cognate transcription factors using chromatin from the liver of double transgenic mice containing either -6A or -6G haplotypes of the hAGT gene. Results of our in vivo ChIP assay showed that HNF-1α binds more strongly to the nucleotide sequence around -1670 region in the chromatin from the liver of transgenic mice containing -6A haplotype of the hAGT gene as compared to the -6G haplotype (Fig. 4, panel a)(compare lanes 1 and 2). Similarly, GR binds more strongly to the -1550 and -217 regions of the hAGT gene promoter in the chromatin of the liver from transgenic mice containing -6A haplotype as compared to -6G haplotype (Fig. 4, panels b and c). Finally, we performed a ChIP assay using antibody against RNA polymerase to examine whether it binds more strongly to the hAGT gene promoter in the chromatin of liver containing -6A haplotype as compared to -6G haplotype. Results of this experiment showed that indeed RNA polymerase binds more strongly to the hAGT gene promoter in the chromatin of liver of transgenic mice
containing -6A haplotype as compared to -6G haplotype (Fig. 4, panel d). Taken together results of this experiment confirmed that RNA polymerase, HNF-1α, and GR have stronger affinity to the hAGT gene promoter in the chromatin obtained from the liver of transgenic mice containing -6A haplotype as compared to -6G haplotype.

**Figure 2-4.** ChIP assay shows that GR, HNF-1α and RNA polymerase bind strongly to the human AGT promoter from liver chromatin of transgenic mice containing -6A haplotype as compared to -6G haplotype. Immuno-precipitated DNA from the two haplotypes in the presence of antibodies against HNF-1α (panel a), GR (panels b and c), and RNA polymerase (panel d) was used to amplify nucleotide sequence containing their respective binding sites as described in Materials and Methods. In all panels (a to d) lanes 1, 2: PCR-amplified product obtained from the ChIP of -6A and -6G haplotypes respectively; lanes 3, 4: PCR-amplified product using input genomic DNA from -6A and -6G haplotypes; lanes 5, 6: PCR-amplified product in the presence of rabbit IgG respectively for -6A and -6G haplotypes. Chip assays were performed in triplicates.
2.16.3. Plasma angiotensinogen level is increased in transgenic mice containing -6A haplotype of the hAGT gene as compared to the -6G haplotype

Equal volume of plasma from control C57 mice and male transgenic animals containing either -6A or -6G haplotype of the hAGT gene was analyzed by Western blot assay along with human plasma as described (19) Quantitation of AGT level was performed after normalization with mouse albumin (Fig. 5). The plasma AGT level was increased by 70% in transgenic mice containing -6A haplotype as compared to -6G haplotype (p=< 0.05). Electrophoretic mobility of hAGT protein in transgenic mice was similar to that of AGT in human plasma.

Fig.2-5. Human AGT level is increased in plasma of double transgenic mice containing -6A haplotype of the hAGT gene as compared to -6G haplotype. Equal amount of plasma
(1μg) from double transgenic mice containing either -6A or -6G haplotype of hAGT gene and hRen gene and from control C57 animals was analyzed by Western blot along with human plasma. Position of molecular weight markers is shown on the left side. Protein levels were normalized with albumin. AGT protein level in transgenic mice was calculated by assuming protein level in C57 mice as one (71).

2.16.4. Blood pressure is increased in transgenic mice containing -6A haplotype of the hAGT gene as compared to -6G haplotype.

A graph showing the mean BP values over a 24hr period of the -6A and -6G haplotypes of transgenic mice (number of animals=6, days of recording =4) is shown in Fig. 6A. Mean BP of control non-transgenic mice was 114mmHg (shown by Con), of transgenic mice containing -6G haplotype was 127mmHg and of transgenic mice containing -6A haplotype was 138 mmHg (n=6)(p<0.05). Taken together, results of this experiment showed that blood pressure of transgenic mice containing -6A haplotype was increased by 11mMHg as compared to transgenic mice containing -6G haplotype of the hAGT gene. Blood pressure of single transgenic mice containing either hAGT or hRen gene was same as that of control C57 mice. Since our experiments so-far suggested that hAGT gene expression is increased in transgenic mice containing -6A haplotype that leads to increased plasma angiotensin-II level and increased blood pressure as compared to transgenic animals containing -6G haplotype, we next examined whether angiotensin receptor blockers reduce blood pressure in these animals. We used losartan as a AT1 receptor blocker and determined the blood pressure by tail-cuff method. Results from this experiment show that losartan reduces the systolic blood pressure in double transgenic
mice containing both -6A and -6G haplotypes of the hAGT gene to or below the non-transgenic baseline level. (Fig. 6B).

**Fig. 2-6.** (A) Blood pressure is increased in double transgenic mice containing -6A haplotype of the hAGT gene as compared to -6G haplotype. Mean of 24 h blood pressure of control (CON) and double transgenic mice containing -6G or -6A haplotype of hAGT gene was measured by telemetry for four days. Each bar represents mean from 6 male animals taken over 4 days. (B) Blood pressure in transgenic mice is reduced by losartan treatment Mean of 24h blood pressure of double transgenic mice containing -6G or -6A haplotypes in the presence (+) and absence (-) of losartan (71).
Fig. 2-7. The figure shows 24 hour systolic blood pressure of transgenic mice with haplotype -6A and haplotype -6G of the hAGT gene. The diurnal rhythm is maintained in both transgenic mice. The difference between the resting SBP of transgenic mice is greater during the time of higher activity (night).
Chapter 3

Hypothesis and Specific Aims of the Research

**Hypothesis:** We hypothesize that increased levels of glucocorticoids will differentially regulate the expression of the hAGT gene in double transgenic mice containing Hap -6A vs. Hap -6G of the gene. We also propose glucocorticoid induced activation of tissue and systemic RAAS with concomitant effects on blood pressure.

**Aim.1.** To examine the effect of dexamethasone on expression of the hAGT gene in the liver and the kidney of transgenic mice containing haplotype -6A or -6G of the hAGT gene

Since, glucocorticoids are involved in the regulation of the blood pressure and are known to induce hypertension in humans and rats (73) and the haplotype -6A of the hAGT gene shows increased glucocorticoid induced promoter activity as compared to haplotype -6G, we propose that treatment with dexamethasone will differentially increase the expression of hAGT in TG mice with haplotype -6A as compared to the haplotype -6G. Therefore, we will examine the effect of dexamethasone on the expression of hAGT gene in the liver and kidney of the double transgenic animals containing human renin
gene and either -6A or -6G haplotype of the hAGT gene. Furthermore, we will also analyze the effect of dexamethasone on the regulation of the endogenous mouse RAAS components.

**Aim.2.** To determine the effect of dexamethasone on the binding of glucocorticoid receptor to the promoter of the hAGT gene in transgenic mice with both haplotypes -6A and -6G.

Since our transfac study shows that: (a) Nucleotide sequence containing -1562C and -1561T has stronger homology with consensus GRE as compared to -1562G and -1561G, and (b) nucleotide sequence containing -217A has stronger affinity for GR as compared to -217G (29), we propose that dexamethasone induced enrichment of GR at the -217, -1561/-1562 region of the hAGT gene will be higher in TG mice with haplotype -6A as compared to that of haplotype -6G. Therefore, to study the effect of DEX on the binding of GR to the hAGT gene, we will evaluate the enrichment of GR at -217 and -1561/-1562 region in the liver and the kidney of the double transgenic mice containing either -6A or -6G haplotypes treated with and without DEX using chromatin immunoprecipitation assay.
**Aim.3.** To examine the effect of dexamethasone on the blood pressure in transgenic mice containing either -6A or -6G haplotypes of the hAGT gene.

We propose that the DEX induced increase in the tissue and/or systemic hAGT levels of the TG mice with haplotype -6A of the hAGT gene may increase the susceptibility to hypertension. In order to detect the effect of DEX on the blood pressure in an in vivo system, we will determine blood pressure in double transgenic mice containing -6A and -6G haplotypes of the hAGT gene and hRen genes using radio-telemetry. We will also evaluate the effect of AT₁R blocker losartan on DEX influenced differences in blood pressure to ascertain the role of differential regulation of the hAGT gene in our transgenic mice with both haplotypes -6A and -6G.
Chapter 4

Material and Methods

4.1. Animal Care and Use

Procedures were approved by the University of Toledo, New York Medical College and Dartmouth Medical College Animal Care and Use Committee (IACUC) and performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

4.2. Transgenic Mice

We have generated transgenic mice containing 180Kbp Bacterial artificial chromosome (BAC) vector (RP11-505D24) harboring the hAGT gene. This BAC has 116 Kb of the 5’-flanking region and 64 Kb of the 3’-flanking region of the hAGT gene (Schematic 4-1). Restriction analysis of the BAC DNA has shown that it contains -6G haplotype of the hAGT gene. We have used a novel and highly efficient galK-based positive/negative selection system for the manipulation of BACs developed by Warming et al (74). We have exchanged 1.8 Kb of the promoter region from a hypertensive subject
containing -6A haplotype into this BAC and generated two BACs containing either -6A or -6G haplotypes. These BACs were thoroughly characterized by restriction analysis and nucleotide sequence analysis and used to generate transgenic mice.

Schematic.4-1. Organization of the hAGT gene in 180Kb BAC. The BAC vector contains 116Kb 5’-flanking region, 54Kb 3’-flanking region and all of the introns and exons of the hAGT gene. Position of important restriction sites is also shown.

The hAGT BAC plasmids were used for microinjection to generate transgenic mice using standard procedure in Dr. Steve Fiering’s lab at the Dartmouth transgenic facility (71). One line of each transgenic mice was selected and analyzed by PCR amplification of hAGT gene from their tail DNA. We amplified 250 bp fragments from 90 Kb upstream regions and 30 Kb downstream region from the hAGT gene to confirm that whole hAGT BAC is inserted in the mouse genome. In addition, we amplified different regions of the hAGT gene from tail DNA of transgenic mice to confirm the presence of BAC DNA containing hAGT gene in these transgenic mice. Transgenic mice
containing -6A and -6G haplotypes of the hAGT gene were analyzed by q-PCR for gene copy number and animals that contained single copy of the hAGT gene were used for future experiments. The presence of the hAGT gene was confirmed by PCR amplification of the tail DNAs of transgenic animals using human AGT gene specific primers. The forward and reverse primers for the amplification of the hAGT gene were CAGCAGTGAAACTCTGC and TTCAGTCATCACCCTGC respectively to produce a 342 basepairs amplification product. Chimeras were bred to C57BL/6 mice and then successfully backcrossed to C57BL/6 for at least seven generations. Copy number of AGT gene in transgenic and C57 mice was determined by q-PCR using human and mouse AGT specific primers and animals with single copy genes were selected for future experiments. Initially four founders from each injection were selected but after gene copy analysis, one line was established from each group for further work. Nucleotide sequence analysis of the hAGT gene promoter from the tails confirmed that transgenic mice with -6A haplotype have variants -6A, -20A, -217A, -532T, -793A, -1074T, -1178G, -1561T, -1562C and -1670A whereas transgenic mice with -6G haplotype have variants -6G, -20A, -217G, -532C, -793G, -1074G, -1178A, -1561G, -1562G and -1670G.


Female transgenic mice containing either -6A or -6G haplotype of the hAGT gene were crossed with male transgenic mice containing human renin gene (PAC-hRen) developed by Sinn et al (75). The genetic background of the PAC-hRen mice was
originally B6SJL (C57BL/6J X SJL/JF2) but have since been backcrossed for at least five generations with C57BL/6J prior to breeding with hAGT mice. The double transgenic mice were genotyped for the human AGT and Ren genes by PCR amplification of the DNA isolated from their tails. The primers for the amplification of hAGT gene were same as used in the analysis of single transgenic mice. The forward and reverse primers for the amplification of hRen gene were CTCTTCGATGCTTCGGATTC and TGGCAGAGTAGGGTGTTCT respectively to produce a 250 bp product. The double transgenic three month old male mice were used for this study.

4.4. Dexamethasone Treatment

Sato et al have shown that glucocorticoid treatment of Wistar rats for five days produced hypertension (76;77). We used the same dose of dexamethasone (Sigma) (2.5μg/mL) in drinking water for 72 hours to examine the effect of glucocorticoids on the expression of the hAGT gene and the blood pressure in transgenic mice. Twelve weeks old double transgenic mice containing haplotype -6A and -6G each were divided into two groups (N=6). Transgenic mice were caged individually. Animals were maintained in a 22 degree C room with a 12-hour light/dark cycle and received standard chow and dexamethasone and vehicle in drinking water ad libitum. For both haplotypes, experiments were performed in the absence and presence of dexamethasone. The background C57/BL6 mice were utilized as non-transgenic controls. No significant difference was observed in the volume of drinking water or dexamethasone solution
consumed by transgenic mice with either haplotype -6A or haplotype -6G and non-
transgenic C57/BL6 mice.

4.5 Quantitative Real-Time RT-PCR

Threshold cycles for three replicate reactions were determined using MxPro-
Mx3005P software (version 4.10), and relative transcript abundance was calculated
following normalization with human GAPDH. Liver and kidney from 8 weeks old male
transgenic mice containing either haplotype -6A or -6G of the hAGT gene and C57
control were harvested following euthanasia and immediately snap frozen in liquid
nitrogen. The extracted tissues were further stored at -80°C until utilized for experimental
purpose. RNA was isolated using RNeasy Plus mini kit (Qiagen). One microgram of
RNA was reverse-transcribed into cDNA using Revert Aid First Strand cDNA Synthesis
Kit (Fermentas) as described in the manufacturer’s protocol. Primers for mouse and
human AGT (cat#PPM04219A, PPH01807A), mouse and human renin (cat#PPM0748A,
PPH07193A) and mouse GAPDH (cat#PPM02946A) genes were purchased from
SuperArray Bioscience Corporation (MD). Following a 95°C incubation for 10 min, 40
cycles of PCR (95°C/30s; 60°C/30s), were then performed using 1µl of cDNA, 50nM
PCR primers and 12.5µl SYBR Green PCR Master Mix in 25 µl reactions. Primers for
mouse 18s rRNA, mouse Beta Actin genes were purchased from Integrated DNA
Technologies (IDT) Inc. Iowa and used to normalize the quantity of mRNA. Gene
expressions were examined using Power SYBR green master mix on ABI 7500 Fast
Real-Time PCR system from Life Technologies.
4.6. Western Blot Analysis

Protein extracts were prepared from the liver and kidney tissues collected from the control C57 mice and hAGT transgenic mice containing hap -6A and hap -6G using Qproteome protein isolation kit (Qiagen). Blood samples were collected by cardiac puncture from mice immediately after exsanguination. Approximately 250µl plasma was collected from each mouse by centrifuging 500ul of blood sample at 3,000 rpm for 15min at 4°C. Protein extracts (25µg) were seperated by SDS-PAGE (12% polyacrylamide) and transferred to 0.45µ PVDF membranes (Millipore, Billerica, MA, US) for 1h. The membranes were incubated with antibody against human angiotensinogen (1:2000) (cat#3249-1, Epitomics, CA). We have previously used an antibody against hAGT which also cross reacts with the endogenous mAGT (Abnova Corporation, Taiwan). Results for liver and kidney were normalized to the mouse β-actin (A2228- Sigma) and that of plasma with mouse albumin (1:10000) (NB600-41532 Novus Biologicals). Finally, blots were developed using an Infrared Imaging System (Odyssey, Li-Cor Biotechnology, Lincoln, Nebraska, US).

4.7. In-vivo Chromatin Immune-Precipitation Assay

The chromatin immune-precipitation (ChIP) assay was performed using the EZ-ChIP assay kit from EMD Millipore, MA, USA. Mice were perfused with normal saline. Liver and kidney was excised and washed in PBS (phosphate buffered saline); minced into smaller pieces; fixed with 1% formaldehyde for 20mins 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 anti-glucocorticoid receptor (anti-GR) 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 as described in the manufacturer’s protocol. The immuno-precipitated DNA (1μl) and the input DNA (1μl) were subjected to 35 cycles of PCR amplification using (a) -314AGT for (CTCAGGCTGTCACACACCTA) as a forward and -6AGT rev (TCTTTCCCCTGGCCGGGTACGAT) as a reverse primer when GR was used to examine the binding of GR at the -217 position of hAGT gene (b) -1757AGT for (CAGGCACAGTGAAACTCTCC) as a forward primer and -1554AGT rev (AGTAACAAGTCCACCTGGAC) as a reverse primer for GR antibody was used to examine the binding of GR across the -1561 and -1562 polymorphic sites. The PCR amplified products were analyzed on 2% agarose gel. The fraction enriched by rabbit IgG was used as a negative control for non-specific binding. A primer set that polymerized DNA fragment on the human angiotensinogen gene with no known glucocorticoid response element was used as non-specific control for GR enriched DNA fragments.
4.8. Measurement of Plasma Angiotensin-II

Plasma angiotensin-II levels were determined by ELISA assay kit purchased from Ray Biotech, Inc. Plasma was collected from C57/BL6 control (non-transgenic) and double transgenic mice containing either -6A or -6G haplotype of the hAGT gene treated with and without dexamethasone. The Angiotensin II concentration in the samples is determined directly from the standard curve as described in the manufacturer’s protocol.

4.9. Blood Pressure Measurement in Mice

Blood pressure (BP) was measured in the conscious state by Radio telemetry (Schematic.4-2). Telemetric probe allows for continuous blood pressure measurements round the clock without distress to the animal after post-surgical recovery. Systolic blood pressure was continuously acquired by implantation of telemetric probe PA-C20 into the aorta via left carotid artery. After one week of recovery from the surgical procedure, blood pressure readings were recorded every ten minutes using Data-Dataquest ART software purchased from Data Science International instrument as described previously. Baseline systolic blood pressure was collected for seven days followed by treatment with DEX. Data for effect of DEX and Losartan on the systolic blood pressure was collected continuously at an interval of 10 minutes for 72 hours. All mice were fed with standard mice chow and had access to water ad libitum. Blood pressure (BP) was measured in the conscious state by telemetry. Radiotelemetric system from Data Science International (St Paul MN, USA) was used for this procedure. Briefly, mice were anesthetized with
ketamine and xylazine (90 and 10mg/kg respectively) and the left carotid artery was isolated. The tip of the telemetric catheter (model TA11PA-C10) was then inserted into the carotid artery and advanced into the aortic arch, with the telemetric device main body positioned into a subcutaneous pocket into the right flank. After one week of recovery from the surgical procedure, BP readings were recorded every ten minutes using Data-Science instrument as described previously (27). Mean BP values were calculated for every hour from the values taken over four days. Animals were then treated with angiotensin receptor blocker, losartan (Sigma Aldrich, USA) (30mg/kg/day), in drinking water. The blood pressure was then measured 24h after losartan treatment.

Schematic.4-2. Radio-telemetric probe implantation in mouse carotid artery
(Courtesy: DataSciences International Website)
4.10. Statistical Analysis

All experiments were conducted with 6 male mice 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).
Chapter 5

Results

5.1. Dexamethasone Induces Expression of the hAGT Gene in the Liver of the Transgenic Mice Containing Hap -6A.

To determine if exposure to glucocorticoids will differentially regulate expression of hAGT gene in Hap -6A and Hap -6G, we treated double transgenic mice containing Hap -6A and Hap -6G with dexamethasone (2.5µg/mL) ad libitum. First, we examined the expression of hAGT in liver of our transgenic mice in response to dexamethasone. As seen in figure 8A, we found that dexamethasone induces mRNA expression of hAGT by 2.5 fold in transgenic mice with Hap -6A (p<0.05) whereas no significant change was observed in hAGT expression in Hap -6G (Figure.5-1A). Endogenous mouse angiotensinogen (mAGT) expression was unaltered in response to dexamethasone in transgenic mice of both haplotypes (Figure.5-1B). Western blot analysis confirmed the induction of hAGT gene in transgenic mice with Hap -6A in response to treatment with dexamethasone (p<0.05) (Figure 5-2A and 5-2B). No significant changes were observed in the mRNA expression of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) in either haplotype -6A or haplotype -6G(data not shown).
Figure 5-1. Expression of the hAGT gene and endogenous mouse AGT in the liver of transgenic mice. Results are shown as mean±SE. Empty bars and solid black bars show baseline and DEX induced gene expression respectively. (5-1A) mRNA expression of the hAGT gene in transgenic mice with Hap -6A is significantly increased in response to...
DEX treatment (5-1B) endogenous mAGT mRNA remains unaltered in TG mice with both Hap -6A and -6G. *p<0.05 against Hap -6G, +p<0.05 against haplotype -6A.

A

Figure.5-2. Western blot showing the expression of the hAGT protein is significantly induced by DEX treatment in the liver of the TG mice with haplotype -6A. (5-2B) Densitometric analysis of hAGT/β-actin shows significant increase in DEX induced expression of the hAGT protein. Lanes 1&2 show bands for haplotype -6G, 3&4 show
bands for haplotype -6A, lane 5 shows absence of the hAGT band in control C57/BL6 mice and lane 6 shows human plasma as positive control. *p<0.05 compared to haplotype -6G, +p<0.05 compared to haplotype -6A.

5.2. Kidney Shows Increased Expression of the hAGT Gene in Transgenic Mice with Hap -6A in Response to Dexamethasone Treatment.

Kidney is a key regulator of the blood pressure. Angiotensinogen is known to be synthesized in kidney and affect blood pressure by modulating pressure natriuresis. Therefore, we analyzed expression of the hAGT gene in renal cortex of our transgenic mice treated with DEX. Using RT-PCR we determined that hAGT mRNA is significantly induced in transgenic mice with Hap -6A (p<0.001). We did not observe any significant changes in mRNA levels of hAGT in Hap -6G (Figure.5-3A). Results for endogenous mAGT mRNA expression were consistent with liver and showed no effect of dexamethasone on its expression in transgenic mice with either haplotype -6A or haplotype -6G (Figure.5-3B). Human AGT protein expression was significantly elevated (p<0.01) in transgenic mice with Hap -6A whereas remained unaltered in Hap -6G after treatment with dexamethasone (Figure.5-4A and 5-4B). Expression of GR and MR is modestly suppressed in response to dexamethasone in transgenic mice with both haplotype -6A and haplotype -6G. As expected, the expression of GR and MR is not affected by the haplotype difference of the hAGT gene in the transgenic mice.
Figure 5-3. Expression of the hAGT gene and endogenous mouse AGT in the kidney of transgenic mice. Results are shown as mean±SE. Empty bars and solid black bars show baseline and DEX induced gene expression respectively. (5-3A) mRNA expression of the hAGT gene in transgenic mice with haplotype -6A is significantly increased in response
to DEX treatment endogenous mAGT mRNA remains unaltered in TG mice with both haplotypes -6A and -6G (5-3B). *p<0.05 compared to Hap -6G, +p<0.05 compared to Hap -6A.

A.

![Western blot showing the expression of the hAGT protein](image1)

B.

![Densitometric analysis of hAGT/β-actin](image2)

**Figure 5-4.** Western blot showing the expression of the hAGT protein is significantly induced by DEX treatment in the kidney of TG mice with haplotype -6A. Densitometric analysis of hAGT/β-actin shows increase in DEX induced expression of the hAGT protein. Lane 1&2 show bands for Hap -6G, 3&4 show bands for Hap -6A, lane 5 shows
absence of the hAGT band in control C57/BL6 mice and lane 6 shows human plasma as positive control. *p<0.05 compared to Hap -6G, +p<0.05 compared to Hap -6A.

5.3. Treatment with DEX Causes an Increase in both hAGT as well as Endogenous mAGT in the Adrenals of Transgenic Mice with Haplotype -6A.

The adrenals express all components of the RAAS. Therefore, we looked at the expression of hAGT and endogenous mAGT in the adrenals of our transgenic mice with and without DEX treatment. We show that DEX treatment causes a significant induction of the hAGT gene in TG mice with Hap -6A (Figure.5-5A). Surprisingly, we also observed higher expression of endogenous mAGT in TG mice containing Hap -6A (Figure.5-5B) at the baseline conditions. Additionally, DEX induced expression of both mAGT and hAGT was observed in TG mice with Hap -6A consistent with our findings in the liver and the kidney. DEX treatment did not affect expression of endogenous mAGT and hAGT mRNA levels in TG mice with Hap -6G. Since adrenal glands play a critical role in synthesis and maintenance of cortisol levels as well as regulation of extracellular fluid volume via secretion of aldosterone, we examined the effect of DEX treatment on regulation of the endogenous aldosterone synthase (Cyp11B2) and 11-beta hydroxylase-1 (Cyp11B1). DEX treatment results in significant suppression of the Cyp11B1 gene irrespective of the genotype of the mice (Figure.5-6A). The expression of the Cyp11B2 gene is increased in the transgenic mice containing Hap -6A of the hAGT gene at the baseline conditions as compared to that to the Hap -6G. However, we only observed a modest increase in the mRNA expression of Cyp11B2 (Figure.5-6B).
Figure 5-5. Q-RTPCR results show induction of both hAGT (A) and endogenous mAGT (B) in adrenals of the TG mice with Hap -6A whereas remains unaltered in Hap -6G. *p<0.05 compared to Hap -6G, +p<0.05 compared to Hap -6A.
Figure 5-6. Expression of the Cyp11B1 and Cyp11B2 genes in the adrenals of TG mice. Results are shown as mean±SE. Empty bars and solid black bars show baseline and DEX induced gene expression respectively. (A) mRNA expression of the Cyp11B1 gene is significantly inhibited in response to DEX treatment (B) mRNA expression of the
Cyp11B2 gene is increased in the TG mice containing Hap-6A of the hAGT gene at the baseline conditions. *p<0.05 compared to Hap-6G, +p<0.05 compared to Hap-6A.

5.4. ChIP Analysis Shows Binding of GR to the Promoter of the hAGT Gene is Increased in the Transgenic Mice with Haplotype -6A on Treatment with DEX.

Since hAGT gene has polymorphisms in the putative GRE sites at -217 and -1561/-1562 region in the promoter and dissimilar nucleotides are present at these locations in haplotype -6A and haplotype -6G. We examined the effect of DEX on the binding of GR to the -217 and -1561/-1562 region of the hAGT gene in transgenic mice with both haplotype -6A and -6G. We first examined the ‘cis-trans’ interactions between GR and chromatin in the liver of our transgenic mice. As seen in (Figure.5-7 and Figure.5-8A) we found that DEX treatment significantly enhances the enrichment of GR to the chromatin around the region of -217 A/G polymorphism in the transgenic mice with haplotype -6A as compared to the baseline. The binding of GR to the -217 region of the chromatin in haplotype -6G was unchanged by DEX. Like the binding at -217 region, DEX increases binding of GR at the -1561/-1562 region in mice with haplotype -6A whereas remains unaffected in mice with -6G (Fig.5-7 and Fig.5-8B). We next analyzed the binding of GR at the promoter of hAGT gene in kidneys of the transgenic mice. Our results in the kidney were consistent with the observation in liver and show a significant DEX dependent increase in the binding activity of GR at both regions including -217 and -1561/-1562 (Fig.5-9 and Fig.5.10). These data suggests that enhanced binding of GR at the promoter of hAGT gene caused by DEX treatment in transgenic mice with haplotype -6A leads to up regulation of hAGT gene in the liver and kidney of these transgenic mice.
Figure 5-7. ChIP assay shows that DEX significantly enhances the enrichment of GR at the -217 and -1561/-1562 region in the promoter of the hAGT gene from liver chromatin of transgenic mice containing haplotype -6A. (A) Immuno-precipitated DNA (Lane 1 and 2 has chromatin from Hap -6G and Lane 3 and 4 from Hap -6A, Lanes 1 and 3 are untreated whereas lanes 2 and 4 are treated with DEX) from the two haplotypes in the presence of antibodies against glucocorticoid receptor (GR) (panel a & d), Input (panel b & e), and IgG (panel c & f) was used to amplify nucleotide sequence encompassing the -217 and -1561/-1562 region respectively as described in Materials and Methods. Panel g shows a PCR reaction for a non-specific (NS) region in the hAGT gene that does not have putative GRE function.
Figure. 5-8. The PCR product of GR enriched DNA relative to input at the -217 (fig.14A) and -1561/-1562 (fig.14B) site of the hAGT gene shows significant increase in the DEX induced GR binding in TG mice with haplotype -6A whereas no difference is observed in TG mice with haplotype -6G. *p<0.05 compared to Hap -6G, +p<0.05 compared to Hap -6A.
Figure 5-9. ChIP assay shows that DEX significantly enhances the enrichment of GR at the -217 and -1561/-1562 region in the promoter of the hAGT gene from kidney chromatin of transgenic mice containing haplotype -6A. Immuno-precipitated DNA (Lane 1 and 2 has chromatin from Hap -6G and Lane 3 and 4 from Hap -6A, Lanes 1 and 3 are untreated whereas lanes 2 and 4 are treated with DEX) from the two haplotypes in the presence of antibodies against GR (panel a & d), Input (panel b & e), and IgG (panel c & f) was used to amplify nucleotide sequence encompassing the -217 and -1561/-1562 region respectively as described in Materials and Methods. Panel g shows a PCR reaction for a non-specific region in the hAGT gene that does not have putative GRE function.
Figure 5-10. The PCR product of GR enriched DNA relative to input at the -217 and -1561/-1562 site of the hAGT gene shows significant increase in the DEX induced GR binding in TG mice with haplotype -6A whereas no difference is observed in TG mice with haplotype -6G. *p<0.05 compared to Hap -6G, +p<0.05 compared to Hap -6A.
5.5. Dexamethasone Treatment Elicits a Significant Increase in the Plasma hAGT and Ang-II levels of Transgenic Mice with Haplotype -6A.

In order to discern the systemic effects of increased tissue angiotensinogen expression caused by dexamethasone treatment, we examined the expression of hAGT in plasma of our transgenic mice. As seen in figure 5-11A and 5-11B, the protein expression of hAGT is significantly increased in transgenic mice containing haplotype -6A o the hAGT gene. DEX treatment has no effect on hAGT expression in plasma of transgenic mice with haplotype -6G. Since catalytic action of renin is the rate limiting step for bioavailability of angiotensinogen, we analyzed expression of human renin (hRen) and endogenous mouse renin (mRen) in the kidney of our transgenic mice treated with and without DEX. There is a general decline in the mRNA expression of both hRen (Figure.5-12A) and mRen (Figure.5-12B) in transgenic mice of both haplotype -6A and haplotype -6G when treated with DEX similar to control C57/BL6 (data not shown). However, transgenic mice with haplotype -6A have most significant attenuation of mRen and hRen mRNA expression in response to DEX treatment. In order to ascertain if inhibition of mRen and hRen alters the systemic angiotensin II levels, we analyzed the angiotensin II (Ang II) levels in the plasma of transgenic mice. Despite the inhibition of hRen and mRen in the kidney of transgenic mice in both haplotype -6A and -6G, we found significantly elevated levels of Ang II in plasma of transgenic mice with haplotype -6A treated with DEX (Figure.5-13) as opposed to no change in transgenic mice with haplotype -6G.
**Figure 5-11.** Western blot shows the expression of the hAGT protein is significantly induced by DEX treatment in the plasma of TG mice with haplotype -6A. Densitometric analysis of hAGT/albumin shows significant increase in DEX induced expression of the hAGT protein (fig.18B). Results are shown as mean±SE. Empty bars and solid black bars show baseline and DEX induced gene expression respectively. Lane 1&2 show bands for haplotype -6G, 3&4 show bands for haplotype -6A, lane 5 shows absence of the hAGT band in control C57/BL6 mice and lane 6 shows human plasma as positive control. *p<0.05 against Hap -6G, +p<0.05 against Hap -6A.
Figure 5-12. DEX treatment causes suppression of the human and mouse renin genes in TG mice containing haplotype -6A of the hAGT gene whereas only a small non-significant reduction is observed in TG mice with haplotype -6G. Results are shown as mean±SE. Empty bars and solid black bars show baseline and DEX induced gene expression respectively. *p<0.05 compared to Hap -6G, +p<0.05 compared to Hap -6A.
**Figure 5-13.** Plasma Ang-II levels (pg/mL) are significantly elevated by DEX treatment in the TG mice containing haplotype -6A of the hAGT gene. Results are shown as mean±SE. Empty bars and solid black bars show baseline and DEX induced gene expression respectively. *p<0.05 against haplotype -6G, +p<0.05 against haplotype -6A.
5.6. DEX Dependent Alteration of the Systolic Blood Pressure (SBP) is Affected by the Nucleotide Differences in the ‘cis’ Elements of Hap -6A and Hap -6G of the hAGT gene.

To determine if the DEX dependent differential regulation of the hAGT gene has an effect on SBP, we implanted radiotelemetry probes in transgenic mice with both haplotype -6A or -6G for accurate and continuous real time analysis of SBP. We observed that transgenic mice with haplotype -6A have significantly increased baseline SBP as compared to haplotype -6G consistent with our published study (71). We next analyzed the effect of DEX on the SBP of both of our transgenic mice with Hap -6A and -6G. Figure 5-14A shows that the elevation in SBP (change/δ) after treatment with DEX (2.5µg/mL) in transgenic mice with haplotype -6A is significantly higher as compared to haplotype -6G after 24, 48 and 72 hours of data collection. We further increased the dosage of DEX to 12.5µg/mL of drinking water and found a stark increase in the SBP of transgenic mice containing haplotype -6A raising it to ~165mmHg whereas the mice with haplotype -6G shows a maximum of ~140 mmHg at this dosage (Figure.5-14B). In order to discern the contribution of DEX induced human angiotensinogen to the increased SBP, we subjected our transgenic mice with 12.5µg/mL DEX along with angiotensinogen receptor type 1 (AT1R) blocker losartan (30mg/kg/day) together in the drinking water. We observed that losartan ameliorates the increased SBP and brings it to the baseline levels in a period of 48 hours even in the presence of DEX (12.5µg/mL) (Figure.5-14B). These experiments establish the susceptibility of haplotype -6A of the hAGT gene to increased SBP on exposure to dexamethasone.
Figure 5-14. The DEX induced elevation of systolic blood pressure is significantly higher at 24, 48 and 72 hours post DEX (2.5µg/mL in drinking water ad libitum) treatment in TG mice with haplotype -6A as compared to that of haplotype -6G (fig. 21A). The SBP of transgenic mice with haplotype -6A shows ~30 mmHg increase as opposed
to ~15 mmHg in haplotype -6G on treatment with 12.5µg/mL DEX in drinking water ad libitum (fig.21B). The DEX induced increase in SBP was alleviated by treatment with AT1R blocker Losartan (30mg/kg/day). *p<0.05 compared to haplotype -6G, +p<0.05 compared to Baseline of the same TG mice and ‡ p<0.05 compared to the DEX treated mice of the same TG mice.
Chapter 6

Discussion

The heritability of hypertension has been estimated up to ~60% however, the existing genetic strategies have explained only about 2-3% of the causality (78;79). The investigation of genetic alleles that increase the susceptibility to hypertension has been perplexing because of the complexity of their homeostatic regulation. The genetic variants that alter the expression of genes involved in the regulation of extracellular fluid volume, cardiac contractility and vascular tone are plausible candidates for predisposing to hypertension. Although several alleles have been linked with hypertension by association studies, the functional mechanisms involved in the pathophysiology of hypertension remains unclear. In this regard, genetic variants of the RAAS genes have undergone extensive scrutiny to analyze genetic susceptibility. RAAS is a key regulator of the blood pressure homeostasis. RAAS maintains the blood pressure by intricate regulation of the feedback mechanisms among various physiological pathways that affect the cardiac output and peripheral vascular resistance. Ang-II is the peptide hormone with potent vasopressor and dipsogenic effects. Ang-II is generated by sequential catalytic action of renin and ACE on the 452 amino acids long polypeptide AGT. The AGT gene has been implicated in hypertension by genetic linkage as well as allelic association
studies.

Human AGT has an A/G polymorphism at -6 position in the promoter and -6A (rs5051) allele is associated with hypertension however molecular mechanisms underlying genetic susceptibility remain unclear. Here we report three additional SNPs in the promoter of the hAGT gene that occur in linkage disequilibrium with rs5051. Based on LD of these SNPs with -6A/G, we characterized two haplotypes of hAGT gene; Haplotype -6A with -6A, -1561T, -1562C and -1670A and Haplotype -6G with -6G, -1561G, -1562G and -1670G. These haplotypes are amenable to differential regulation of the hAGT gene by transcription factors, including the GR. Using a 1.8 kb reporter construct, our lab has shown increased DEX induced promoter activity in the haplotype -6A as compared to haplotype -6G (80). To characterize tissue specific regulation of the hAGT gene and its effect on the regulation of the blood pressure in an in vivo situation, our lab has generated double transgenic mice containing either Hap -6A or Hap -6G along with the human renin gene. 180Kb long BAC DNA containing 116 Kb of the 5’-flanking region, all exons and introns, along with 54 Kb of the 3’-flanking region of the hAGT gene was used to generate TG mice. The reason behind using BAC DNA was that since it contains 116Kb of the 5’-flanking region, it will have all the cis-acting DNA elements required for tissue and hormone specific expression of the hAGT gene. Since hAGT gene is not cleaved by mouse renin, we generated double transgenic mice containing human renin gene and either haplotype -6A or -6G of the hAGT gene.

We have reported that the hAGT mRNA level is increased by about 2.5 fold in the liver of male double transgenic mice containing Hap -6A as compared to Hap -6G. The
hAGT protein level is also increased in the liver of transgenic mice containing -6A haplotype by about 1.6 fold as compared to the liver of TG mice with -6G haplotype. In addition, the in vivo ChIP assay showed that the GR has stronger affinity to the chromatin obtained from the liver of transgenic animals containing -6A haplotype as compared to -6G haplotype. Our lab also determined that the plasma hAGT level is increased by about 1.7 fold in transgenic mice containing -6A haplotype of the hAGT gene as compared to the -6G haplotype. Finally, our lab reported that the systolic blood pressure is increased by 11 mmHg in male double transgenic mice containing hRen gene and -6A haplotype of the hAGT gene as compared to -6G haplotype. The 24 hour blood pressure data acquired by radio-telemetric system shows that the diurnal rhythm of the blood pressure is maintained in both of our TG mice. The maximum difference in the blood pressure of our TG mice is observed during the active night hours. Finally, the fact that this increase in the blood pressure is due to increased level of angiotensin-II is shown by our experiments that angiotensin receptor blocker (losartan) reduced the blood pressure in transgenic mice. Since the alterations of the GR and HNF-1α binding sites in our TG mice with Hap -6A and Hap -6G resulted in the baseline differences in the expression of the hAGT gene and the blood pressure, we hypothesized that the exposure to excess amount of glucocorticoids will increase the susceptibility of TG mice with Hap -6A to hypertension.

Glucocorticoids are widely used in current medical regimes to alleviate symptoms of allergy, asthma and organ transplantation. Glucocorticoids are also known to increase blood pressure. However, the mechanisms underlying glucocorticoid induced hypertension remain unclear. Promiscuous activation of MR has been accounted for elevation of blood pressure by sodium water retention. In contrast, specific
glucocorticoids that do not interact with MR are also reported to increase blood pressure (51). This suggests that genes that are regulated by GR and can affect the vascular tone may be involved in modulation of blood pressure. In this regard, the hAGT gene could be a plausible candidate for increase of blood pressure in response to glucocorticoids. There are multiple putative glucocorticoid response elements across 100kb region of the hAGT gene as shown in schematic.9. SNPs in these GRE elements may alter hAGT expression and thereby affect the blood pressure phenotype. The SNPs at the -217, -1561, -1562 and -1670 sites in the Hap -6A modulate the binding sites for the transcription factors HNF-1α, C/EBP and GR in the promoter of the hAGT gene. Exposure to glucocorticoids may therefore increase the transcription of the hAGT gene in TG mice with Hap -6A. Dexamethasone is a specific agonist for GR and has been widely used as a pharmacological intervention to study the effects of GR activation. In order to ascertain if haplotype -6A of the hAGT gene increases the susceptibility to hypertension on exposure to glucocorticoids, TG mice with Hap -6A and Hap -6G were treated with DEX (2.5µg/mL) in drinking water ad libitum.

Previous in vitro and in vivo studies have shown that glucocorticoids play an important role in the expression of AGT gene in the liver (81). Glucocorticoid treatment increases blood pressure in humans (82) and increased cortisol level is a marker for human hypertension (83). Since liver is primary contributor to the plasma AGT, we examined the regulation of the hAGT gene in our transgenic mice on exposure to DEX (2.5µg/mL in the drinking water ad libitum). Here we report that the DEX treatment caused a significant induction of the hAGT gene in TG mice with Hap -6A. We did not observe any alterations in the hAGT gene in the liver of the TG mice with Hap -6G. At
this dosage of DEX, the mRNA expression of endogenous mAGT was not affected in the liver. The SNPs in the promoter of Hap -6A creates a strong binding site for GR, HNF-1α and C/EBP. Glucocorticoid treatment affects the regulation of genes that contain C/EBP binding sites (84;85) by increasing the synthesis of C/EBPβ and C/EBPδ via GR in the hepatocytes (86). Also, GR and HNF-1 synergistically increase DEX induced expression of genes (87). Therefore, it is plausible that glucocorticoid induced activation of the transcription factors including GR, C/EBPβ, C/EBPδ and HNF-1α in the hepatocytes have an overall cumulative effect on the expression of the hAGT gene in the liver of transgenic mice containing Hap -6A.

In order to determine the effect of glucocorticoids on renal and systemic RAAS, we next examined the regulation of the hAGT gene in the kidneys of both TG mice treated with DEX. The de novo AGT synthesized by the local RAAS in the kidney has been implied in hypertension by modulation of pressure natriuresis. Consistent with our results in the liver, DEX induced increase in the hAGT was observed only in the kidneys of TG mice with Hap -6A. No change was observed in the mRNA expression of endogenous mAGT gene. We know that the HNF-1 binding site is required for glucocorticoid induced transcription of insulin-like growth factor binding protein-1 (88) and PEPCK gene in the kidney (89). We propose that the DEX dependent increased promoter activity at the HNF-1α site may be involved in the up-regulation of the hAGT gene in the kidneys of TG mice with hap -6A. The differential regulation of the hAGT gene in the kidneys of our transgenic mice implies a significant renal dependent modulation of the blood pressure homeostasis.
Local RAAS in the adrenal glands also plays an important role in the regulation of the blood pressure by secretion and maintenance of the plasma corticosteroid levels. The corticosteroids including cortisol and aldosterone regulates blood pressure by multiple genomic and non-genomic effects. Cortisol is a glucocorticoid hormone secreted in the zona fasciculata of the adrenal cortex under the regulation of hypothalamus-pituitary-adrenal (HPA) axis. The generation of cortisol is triggered in response to stress and higher physical activity. Although cortisol is a potent ligand for the GR, it also causes activation of the MR. Adrenal glands express AGT both in the medulla and cortex regions (90). Apart from systemic, local Ang-II is also implicated in the regulation of aldosterone and corticosterone via angiotensin receptors in a number of rodent models (91). The regulation of the hAGT gene and other components of RAAS may be modulated in our TG mice at the resting state. Therefore, we analyzed the expression of the hAGT gene in the adrenals of our TG mice. Here we report that the expression of the hAGT gene is significantly higher in the TG mice with Hap -6A as compared to the Hap -6G at the baseline conditions. Treatment with DEX causes a significant induction of the hAGT gene in TG mice with Hap -6A whereas no change was observed in the Hap -6G. Contrary to our results in the liver and kidney, the mRNA levels of endogenous mAGT are also significantly increased in the TG mice with Hap -6A. Adrenals have complete RAAS system with all its components synthesized de novo in the adrenals. Also, increased Ang-II levels is known to increase the stability of AGT mRNA (92). Since mAGT is induced only in TG mice with Hap -6A and not Hap -6G, we assume that increased levels of local Ang-II in the adrenals may be contributing to the stability of mAGT mRNA. We next analyzed the effect of DEX treatment on the regulation of
corticosteroids aldosterone synthase (Cyp11B2) and 11-β hydroxylase (Cyp11B1) genes in the adrenals. Consistent with previous studies that have showed inhibition of Cyp11B1 gene on treatment with DEX (93), we observed a decline in the Cyp11B1 mRNA levels in both our transgenic mice. As expected, we did not observe any haplotype specific differential regulation of Cyp11B1 in our TG mice with or without DEX treatment. Ang-II is a physiological regulator of the Cyp11B2 gene expression in the zona glomerulosa of the adrenal (94-96) and directly contributes to the plasma aldosterone levels. The adrenals in the TG mice with Hap -6A showed elevated levels of Cyp11B2 mRNA as compared to the Hap -6G. The increased Ang-II levels in the TG mice with Hap -6A may be inducing expression of the Cyp11B2 gene at the resting levels as opposed to unaltered levels in TG mice with Hap -6G. Increased aldosterone levels in the plasma results in Na/H retention and consequential increase in the extracellular fluid volume and the blood pressure via activation of MR receptors. Activated MR may also bind to the GRE sites in the promoter of the hAGT gene resulting in a further increase of the hAGT transcriptional activity. Although DEX caused increased expression of the hAGT and mAGT gene in the adrenals of the TG mice with Hap -6A, we did not observe a significant difference in the expression of Cyp11B2 gene on treatment with DEX. Treatment with DEX is known to suppress the signaling activity of the HPA axis. It is possible that the inhibition of the HPA axis and activation by elevated Ang-II have an overall counterbalancing effect on the regulation of the Cyp11B2 gene in TG mice with Hap -6A on treatment with DEX. Chronic administration of DEX to these transgenic mice may provide insights on effect of increased glucocorticoid levels on the regulation of Cyp11B2 gene and plasma aldosterone levels in subjects with Hap -6A of the hAGT gene.
The in vitro studies in our lab showed that the SNPs in the promoter of the hAGT gene in TG mice with Hap -6A modulates the binding sites of the glucocorticoid activated transcription factors resulting in the increased promoter activity as compared to the Hap -6G at the resting state (71). We hypothesized that the exposure to increased glucocorticoid levels will further accentuate the transcriptional regulation of the hAGT gene in the TG mice with hap -6A incurring susceptibility to hypertension. In order to ascertain this hypothesis in an in vivo situation, we utilized the chromatin immunoprecipitation assay to analyze the enrichment of GR on the GR dependent transcription factor binding sites encompassing the proposed functional SNPs including -217G/A and (-1561T/G and -1562C/G) sites in both out TG mice with Hap -6a and Hap -6G. Our ChIP analysis shows that the DEX induced binding of GR at the -1561 and -1562 site is significantly higher in the liver and the kidney of the TG mice with hap -6A demonstrating the functional role of -1561T and -1562G in the differential regulation of the hAGT gene. The enrichment of GR at the -217 A/G site close to the promoter is significantly higher in the liver and the kidneys of the TG mice with Hap -6A on treatment with DEX. We did not observe any changes in recruitment of GRs at these polymorphic sites in TG mice with Hap -6G by DEX. The elevated enrichment of GR at the hAGT promoter in TG mice with Hap -6A in response to DEX may also facilitate binding of glucocorticoid activated transcription factors including C/EBPβ, C/EBPδ and HNF-1α. Therefore we believe that the glucocorticoid activated promoter activity of the hAGT gene leads to its higher expression in the liver and kidney.

Whether increased expression of the hAGT gene in the liver, kidney and adrenals have an effect on the plasma concentrations of hAGT and Ang-II is critical to understand
physiological repercussions of elevated glucocorticoids in TG mice with Hap -6A. Our immuno-blotting assay for measurement of plasma hAGT level demonstrates significant DEX induced expression in TG mice with Hap -6A as opposed to unchanged levels in Hap -6G. The catalytic action of renin on the AGT is essential for synthesis of decapeptide Ang-I which is subsequently cleaved by ACE to generate the potent effector octapeptide Ang-II. The cleavage of AGT by renin is a rate limiting step in the RAS and determines the plasma Ang-II levels. The concentrations of hAGT levels in the plasma are lower than the michaelis constant (Km) for renin (54) implying that the elevation of the plasma hAGT levels leads to increased plasma Ang II concentrations. Using EIA to detect the plasma Ang-II levels in our TG mice, we show here that the plasma Ang-II levels are significantly elevated in TG mice with Hap -6A after DEX treatment. DEX treatment did not significantly affect the Ang-II levels in TG mice with Hap -6G. Activated RAAS system has been shown to maintain the blood pressure homeostasis by feedback mechanisms via regulation of renin in normal physiological conditions. Therefore we analyzed the expression of renin in the kidneys of our TG mice with and without DEX treatment. We show here that both hRen and endogenous mRen were down regulated in TG mice containing Hap -6A at the resting conditions. Treatment with DEX causes suppression of the renin in the kidneys of TG mice with both haplotypes. However, the attenuation of renin in TG mice containing Hap -6A is clearly significant as opposed to that of small decrease observed in Hap -6G. We believe that the increased tissue and plasma Ang-II levels activate the negative feedback loop of the RAAS consequentially suppressing renin in the kidneys of TG mice with Hap -6A. Although suppression of renin in the kidneys inhibits generation of the plasma Ang-II, reports of
higher plasma Ang-II levels associated with hypertension (4;97) have demonstrated that the feedback loop is not absolute. Also, the classical gene copy number experiments in transgenic mice by Kim et al showed that the systolic blood pressure increases by 8mmHg with addition of subsequent copy number (98). Therefore we believe that the increased Ang-II levels in the plasma and local tissues may be predisposing subjects with Hap -6A of the hAGT gene to hypertension.

Excess glucocorticoids in the plasma because of pathophysiological disorders like Cushing’s syndrome or chronic pharmacological interventions such as dexamethasone is known to increase the blood pressure (49). Increased glucocorticoid levels have multiple genomic and non-genomic effects. Excess glucocorticoids activate the MR to increase the sodium water retention resulting in hypertension. However treatment with MR blockers does not alleviate cortisol induced hypertension in humans (99). Also, treatment with specific GR antagonist RU486 alleviates symptoms of glucocorticoid induced hypertension in rats (100) whereas MR blocker spironolactone failed to do so. The mechanisms underlying the pathophysiology of glucocorticoid induced hypertension remain unclear. We know that the genomic effects of glucocorticoid excess are mediated by the GRs that bind to the GREs and modulate gene expression. We propose that the differential induction of hAGT gene by GR on exposure to higher levels of glucocorticoids as a plausible mechanism for glucocorticoid induced hypertension in subjects with allele Hap -6A of the hAGT gene. Our experiments on the regulation of the hAGT gene in transgenic mice model treated with DEX has demonstrated that the hAGT gene is significantly induced in TG mice containing Hap -6A showing elevated Ang-II levels whereas TG mice with Hap -6G did not show any
change. In order to ascertain the physiological relevance of DEX dependent differential regulation of the hAGT gene in various tissues and systemic Ang-II levels, we measured the systolic blood pressure (SBP) of our male TG mice. We did not observe changes in SBP at 0.25µg/mL dosage of DEX. Here we have shown that the DEX (2.5µg/mL in drinking water *ad libitum*) treatment leads to significantly elevated SBP in TG mice with Hap -6A as compared to that of Hap -6G. Therefore all our gene expression, ChIP analysis and Ang-II measurement experiments were performed at 2.5µg/mL in drinking water *ad libitum*. It is noticeable that after 72 hours of treatment with DEX, TG mice with Hap -6A shows a significant increase of 12mmHg in the SBP as compared to the vehicle. As expected, the change observed in Hap -6G is only about ~5mmHg similar to background C57/BL6 mice at this dosage. In order to examine if the effect of DEX on the SBP of our TG mice was dose-dependent, we increased the dosage of DEX to 12.5µg/mL of drinking water. Exposure to higher dose of DEX caused a significant rise of about 15mmHg SBP in the TG mice with Hap -6G. We believe that the increase in SBP of the TG mice with Hap -6G on treatment with higher dose DEX may be because of the DEX induced activation of the MR. Activated MR increases the expression of epithelial sodium channel (ENac), Na+/K+ pump and serum and glucocorticoid induced kinase1 (SGK-1) resulting in sodium water retention that leads to increase in the extracellular fluid volume and consequential elevation of the blood pressure. The differential regulation of the hAGT gene in TG mice with Hap -6A is still evident showing a striking increase in the SBP (about 33mmHg) as compared to the vehicle. We showed that the difference in the resting SBP of our male TG mice was ~11mmHg with TG mice containing Hap -6A showing a SBP at 137mmHg as opposed to 126mmHg in TG mice.
with Hap -6G. Treatment with 12.5µg/mL DEX aggravates the increase in the SBP of the TG mice containing Hap -6A raising it to 165 mmHg about 25 mmHg higher than the TG mice with Hap -6G at the same dose. Our results thus dissect the effect of MR activation on the blood pressure at higher doses in TG mice of both haplotypes from the GR mediated differential activation of the RAAS showing a differential rise of the SBP in TG mice with Hap -6A. To demonstrate that the rise in the SBP on DEX treatment is mediated via the activation of RAAS system, we administered AT$_1$R blocker losartan (30mg/kg/mice) to our TG mice on the high dose DEX of 12.5µg/mL in the drinking water. Here we show that losartan averts the DEX dependent rise in the SBP of our TG mice and brings it to the baseline levels in 48 hours even in the face of the DEX treatment. These results show the role of activated RAAS, in differential elevation of the SBP in TG mice with Hap -6A as compared to the TG mice with hap -6G, via up regulation of the hAGT gene on exposure to excess glucocorticoids.

The DEX dependent elevation of the SBP in our TG mice is most likely mediated by the increased plasma Ang-II modulating the vascular tone and eventually SBP. However, the up regulation of the hAGT gene in the kidneys of TG mice with Hap -6A could be an independent contributor to the increased blood pressure of our TG mice by modulation of the pressure natriuresis (101). Besides, the activation of local renal RAAS has been shown to be unaffected by the regulatory feedback mechanisms of systemic RAAS and contribute to chronic hypertension (102) as well as renal pathologies including chronic kidney disease (103). Also GR is extensively expressed in the renal tissue distributed across the glomerulus, proximal convoluted tubules and thick ascending limb of the nephron(104) however, its role in the renal physiology is yet to be
understood. Our ChIP analysis has shown the binding of GR to the hAGT promoter is increased in the kidneys of the TG mice with Hap -6A on treated with DEX. Therefore, we propose that the GR activated by excess glucocorticoids up regulates the expression of hAGT and Ang-II bioavailability in the kidneys of TG mice with Hap -6A and affects their blood pressure.
Chapter 7

Future Prospects

Stress whether physiological or psychological may cause up regulation of the glucocorticoid levels and thereby trigger glucocorticoid receptor (GR) expression and activity in various tissues. The analysis of acute and chronic stress model experiments on our TG mice will help us dissect the effect of physiologically activated GRs on the regulation of the hAGT gene and the blood pressure. The use of specific GR antagonist RU486 (mifepristone) and aldosterone antagonist (Spironolactone) with and without DEX treatment will help us explore the involvement of DEX dependent activation of GR vs. MR in the glucocorticoid induced hypertension. Exposure to high salt results in the activation of MR. Our TG mice model subjected to high salt diet with and without spironolactone can be utilized to study the effect of MR on the regulation of the hAGT gene and the blood pressure.

Glucocorticoid treatment rapidly increases the synthesis and activity of transcription factors C/EBPβ and C/EBPδ in adipocytes which plays a crucial role in the expression of adipogenic genes (105). Obesity is associated with hypertension (106;107) and it is estimated that between 65% and 75% of cases of hypertension could be
attributed to obesity (108). The angiotensinogen generated by adipocytes is second largest contributor to the systemic AGT. Over-expression of the AGT gene in the adipose tissue has been shown to increase plasma AGT level and cause hypertension in transgenic mice (30). Also, adipocyte derived AGT is directly implicated in obesity induced hypertension (109). Our transgenic mice model fed on high fat diet with and without mifepristone and spironolactone can be utilized to investigate if there is synergistic effect of increased GC levels and obesity on regulation of hAGT and blood pressure.

We have reported DEX dependent differential regulation of the hAGT gene in the kidneys of our TG mice. Activation of the local renal RAAS is implied in hypertension and renal diseases. The analysis of renal structure and physiology in our transgenic mice on chronic physiologically elevated glucocorticoids utilizing experimental stress models may help us understand the contribution of kidney to the hypertensive phenotype associated with Hap -6A of the hAGT gene.
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