FUNCTIONAL IMPACT OF ANGIOTENSINOGEN SPECIFIC DOMAINS ON ANGIOTENSIN II-MEDIATED FUNCTIONS

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FUNCTIONAL IMPACT OF ANGIOTENSINOGEN SPECIFIC DOMAINS ON ANGIOTENSIN II-MEDIATED FUNCTIONS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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Lexington, Kentucky

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and Dr. Alan Daugherty, Professor of Physiology
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2020

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Angiotensinogen (AGT) is the only substrate for all angiotensin peptides in the renin angiotensin system (RAS). Cleavage of AGT by renin is a rate-limiting step of angiotensin peptides productions in the RAS and regulates angiotensin peptides-associated pathophysiological functions. Only ten N-terminal residues are cleaved by renin and the functions of remaining part of AGT protein, which is called des(angiotensin I)AGT, remain unclear. Despite of pivotal roles of AGT in the RAS, studies related to how AGT is metabolized and how des(angiotensin I)AGT regulates AGT functions or AngII-mediated functions are limited.

Renin cleavage of AGT shows species specificity. It has been reported that residues in N-terminus of AGT surrounding renin cleavage site play important roles of affecting renin activity in vitro by using synthetic peptides. However, there is no in vivo evidence showing the effects of N-terminal residues in renin cleavage of AGT and consequently AngII-mediated functions, such as blood pressure and atherosclerosis. In this study, we compared expressions of AGT proteins by adeno-associated viral (AAV) vectors encoding human AGT or mouse AGT with Leu11Val and Tyr12Ile mutations which mimics two residues at the same position in human AGT to determine whether substitution of these residues regulated AngII-mediated functions. All mice were LDL receptor⁻/⁻ mice. Hepatocyte-specific AGT deficient (hepAGT⁻/⁻) mice were injected with AAVs encoding null AAV, human AGT AAV or mutated mouse AGT (L11V;Y12I). hepAGT⁺/⁺ littermates injected with null AAV as positive controls. Expression of human AGT did not affect endogenous mouse AGT expression, blood pressure and atherosclerosis in hepAGT⁻/⁻ mice. In a subsequent study, expression of L1V;Y12I AGT significantly increased plasma AGT concentration, blood pressure and atherosclerosis, showing that replacement of L11 and Y12 to V11 and I12, respectively, in mouse AGT does not affect renin cleavage, blood pressure, and atherosclerosis in LDL receptor deficient mice.

AGT has been reported to have both AngII-dependent and -independent functions. In our previous publication, we found two highly conserved regions, the β-sheet and loop region, on the surface of AGT which are distal to renin interacting face, and showed that these two highly conserved regions might have renin-independent roles. Moreover, we demonstrated that AGT and megalin interactions predispose animals to atherosclerosis. Thus, in this study, we investigated whether these two highly conserved regions affect AngII-mediated functions thorough influencing AGT interaction with megalin. Firstly, we determined the roles of the loop region in the interaction of AGT with megalin in vitro. All mice were LDL receptor⁻/⁻ background. However, repopulation of mouse AGT with single nucleotide mutation on the loop region, triple nucleotide mutations on the β-sheet or the loop region, and replacement of whole conserved sequences in the loop region with a GA linker did not change blood pressure and atherosclerosis in hepAGT⁻/⁻ mice, which is associated with plasma AGT concentration. Interestingly, mutations on both the β-sheet
and loop region inhibited plasmas AGT level, blood pressure and atherosclerosis, showing the β-sheet and loop region synergistically regulate AGT production and consequently affect AngII-mediated functions in hepAGT−/− mice.

Taken together, residues in AGT have different biological effects which regulates angiotensin-associated functions.

KEYWORDS: Angiotensinogen, Angiotensin II, Atherosclerosis, and Blood Pressure.

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CHAPTER 1. LITERATURE REVIEW – CRITICAL ROLES OF ANGIOTENSINOGEN IN ANGII-MEDIATED FUNCTIONS

This chapter provides an introduction to relevant concepts of my research works in this dissertation. This dissertation discusses the possible roles of specific amino acids of AGT in AGT metabolism and AngII-mediated functions.

1.1 Introduction

The renin angiotensin system (RAS) is a well-known hormone system regulating body fluid and blood pressure [1, 2]. Angiotensinogen (AGT), the only substrate of the RAS to produce all angiotensin peptides, is converted into angiotensin I (AngI) by renin. AngI is then converted to angiotensin II (AngII) by angiotensin-converting enzyme (ACE). AngII is an octapeptide which mediates angiotensin II receptor type 1 (AT1)-mediated physiological and pathological functions. (Figure. 1.1). Human AGT is 452 amino acids long and mouse AGT is 453 amino acids long. Since the pivotal roles of AGT in the RAS, AGT regulates AngII production and AngII-mediated functions, such as atherosclerosis and hypertension.
Atherosclerosis, a disease caused by plaque building-up inside arterial wall, is a leading risk factor of many cardiovascular diseases [3]. It has been reported by many groups that the RAS is involved in the pathogenesis of atherosclerosis [4-6]. In the RAS, AngII is a major factor contributing to vascular pathology by direct and indirectly affecting vascular wall and various types of cells (Figure 1.2) [6-8]. AngII induces oxidative stress [9-11], inflammatory cytokines and adhesion molecules production [12, 13], LDL oxidation and uptake [14, 15], and endothelial dysfunction [16]. For example, AngII infusion (500 ng/kg/min) for 28 days increased cholesterol accumulation in atherosclerotic lesions in LDL receptor\(^{-/-}\) mice [17]. AngII infusion (1000 ng/kg/min) for 28 days increased macrophage accumulation in atherosclerotic lesions and promoted severity of atherosclerosis in apoE\(^{-/-}\) mice [6]. Inhibition of AngII production by blocking renin [18-20] or ACE [21-24] attenuates atherosclerosis. In addition, AT1 receptor antagonism by AT1 receptor blocker or genetic knockout attenuates atherosclerosis in animals and human.
In animals, AT1 receptor antagonists, such as losartan [25-27], valsartan [28, 29] and irbesartan [30, 31], olmesartan [32] and telmisartan [33], significantly decreased intimal lesions in hypercholesterolemia-induced atherosclerosis in monkeys, rabbits or mice. AT1A receptor deficiency significantly reduces hypercholesterolemia-induced atherosclerosis in LDL receptor−/− mice [34, 35], which associates with the decrease in hypercholesterolemia-enhanced systemic AGT and angiotensin peptides [35]. AT1A receptor deficiency also attenuates vascular dysfunction and atherosclerosis in apoE−/− mice [36, 37]. In human, irbesartan attenuates inflammatory markers in premature forms of atherosclerosis [38] and inhibits MMP-induced plaque rupture by blocking COX-2/mPGES-1 expression [39]. Losartan [40, 41] and olmesartan [42] significantly reduce intima-media thickness in patients with hypertension. Taken together, AngII-AT1 receptor pathway regulates and promotes the formation of atherosclerosis.

**Figure 1.2 Renin angiotensin system contributes to atherosclerosis.**

Reports show that the RAS elevates blood pressure in human and animals. In human, drug interventions to treat hypertension are well established. However, hypertension-related cardiovascular complications, such as left ventricular hypertrophy (LVH), are commonly seen in hypertensive patients. Angiotensin II receptor blockers
(ARBs), which have been widely used for lowering blood pressure in hypertensive patients, show beneficial effects on hypertension-related cardiovascular complications. For example, losartan not only reduced blood pressure but also prevented more cardiovascular morbidity and death than atenolol, a β-blocker, did [43, 44]. Losartan reduced blood pressure, protected arterial structure as well as endothelial functions [45], and regressed myocardial fibrosis [46] in hypertensive patients. To better understand the roles of the RAS components in the regulation of blood pressure, inhibition of the RAS components was studied in animal models. For example, losartan, an AT1 receptor antagonist, blunted the blood pressure increase caused by diet-induced obesity in male Sprague-Dawley rats [47]. When administered either acutely or chronically, enalapril, an angiotensin converting enzyme inhibitor, decreased blood pressure in rats [48]. Angiotensin type 1a (AT1a) receptor−/− mice showed significantly decreased blood pressure [49].

AGT is the only precursor of AngII. There is also evidence that AGT is associated with AngII-dependent vascular pathology and blood pressure regulation. In atherosclerosis, single nucleotide polymorphisms of AGT are associated with coronary atherosclerosis. For example, T704C [50], T174M [51, 52] and M235T [51, 53-55] of AGT are associated with coronary atherosclerosis in different populations. In addition, deficiency of plasma AGT significantly decreased atherosclerotic lesions in hepatocyte-specific AGT deficient mice [56-59]. hypoAGT mice, which had greatly reduced AGT mRNA abundance in all tissues, also showed significant decrease in atherosclerosis and blood pressure [59]. Antisense oligonucleotides targeting AGT attenuated high blood pressure, atherosclerosis and many other diseases [60-62]. In hypertension, overexpression
of rat AGT increased plasma AGT concentration and blood pressure in mice [63]. Whole body AGT knockout mice, which had severe impact on neonatal survival rate and renal functions, showed hypotension [64]. Infection of adeno-associated viral vector encoding AGT antisense oligonucleotide in spontaneously hypertensive rats significantly decreased blood pressure with a short-term [65, 66] or a long-term effect [67]. Small interfering RNAs (siRNAs) targeting hepatic AGT reduced blood pressure [68, 69], and inhibited preeclamptic phenotypes [70]. In summary, these findings reveal the important roles of AGT in AngII-mediated functions.

### 1.2 Molecular characteristics of AGT

#### 1.2.1 AGT gene and protein structure

AGT is present throughout vertebrate evolution and is conserved from lampreys to human [71]. Human AGT, which is localized on chromosome 1 and consists of 5 exons [72, 73], is 485 amino acids with a 33-amino acids signal peptide [74]. Mouse AGT, which is located on chromosome 8 and consists of 6 exons [75, 76], is 477 amino acids including a 24-amino acids signal peptide. AngI, 10 N-terminal amino acids, is cleaved from AGT and the remaining part of AGT which is called as des(AngI)AGT.

#### 1.2.2 Effects of conserved and non-conserved residues of AGT on AGT metabolism

Amino acids determine gene expression and protein functions. There are many studies reporting amino acids of AGT, such as genetic variants and formation of disulfide bond, affect AGT expression and production as well as AGT-associated diseases.
1.2.2.1 Genetic variants

Genetic variants have been showed to regulate AGT expression and associate with hypertension and other diseases. Two variants of the AGT gene, M235T and T174M, have been extensively studied and shown to be associated with hypertension [77-82] and other cardiovascular diseases [83-85] in certain populations, such as Japanese and Caucasians. For example, M235T of AGT predisposed to hypertension and associated with cardiovascular risk factors in Japanese [80], and also associated with pre-eclampsia [86]. M235T of AGT was independently and significantly associated with coronary heart diseases in Caucasians [83]. A meta-analysis which included 127 studies published between January 1992 and March 2002 showed the association of M235T of AGT with plasma AGT concentration and risk of hypertension [87]. T174M of AGT was significantly associated with systolic blood pressure only in men in the Hutterite Brethren, a North American religious genetic isolate [88]. In addition, other gene polymorphisms of AGT, such as T704C are associated with cardiovascular diseases, including coronary atherosclerosis [50]. However, some studies showed that M235T or T174M of AGT might not be a risk factor for hypertension or coronary heart disease in some ethnic populations, such as Indian [89], East Anglian subjects of the United Kingdom[90], Chinese [91], African Caribbean [92], and African American [93].

Genetic variants have been found to contribute to AGT production, which might be the reason causing hypertension and cardiovascular diseases. It was reported that higher frequency of T235 of AGT in black children had higher plasma AGT concentration than in white children [94]. Also, higher plasma AGT concentration was found in female
carrying M235T than in male in Lake Salt City [95]. To understand the causality between AGT gene with plasma AGT concentration and hypertension, genetic mice with zero- to four- copy of mouse AGT gene in normal chromosomal location were generated and tested. Plasma AGT concentration was gradually increased in a copy number-dependent manner, which positively related to blood pressure [96].

These studies show that genetic variants of AGT, such as M235T and T174M determine AGT production and downstream AngII-regulated blood pressure as well as cardiovascular diseases based on gene frequency, gender, ages, and ethnics.

1.2.2.2 Disulfide bond

There are four cystines in AGT and two cystines (Cys18-Cys138 in human and Cys18-Cys137 in mouse) forming a disulfide bond are conserved among species [97, 98]. Cys18-Cys138 disulfide bond has been reported to involve in regulation of structure of N-terminus of AGT [97]. Zhou et al. (2010) reported that Cys18-Cys138 disulfide bond facilitated active site cleft of renin binding on N-terminus of AGT and consequently enhanced cleavage of AGT to release AngI by protein crystal structure analysis [99]. Moreover, they found that percentage of reduced form of AGT decreased which means oxidized AGT increased in pregnant women with preeclampsia, showing redox-switch of AGT regulates AngII production and hypertension. Rahgozar et al. [100] used another method, ELISA to measure plasma total and reduced AGT and confirm Zhou et al. results done by Western blot analysis. Rahgozar et al. in 2015 showed the same result that reduced AGT significantly decreased in pregnant women with preeclampsia. Most recently, Dahabiyeh et al. (2020) confirmed decreased ratio of oxidized AGT to total AGT in
pregnant women with preeclampsia by a mass spectrometry-based method [101]. However, repopulation of mutated AGT with lacking Cys18-Cys137 disulfide bond in hepatocyte-specific AGT deficient mice did not affect blood pressure and atherosclerosis, showing that Cys18-Cys137 disulfide bond formation is not essential for AgnII production and AngII-mediated functions in mice [56]. In summary, disulfide bond formation might determine AGT metabolism and predisposes to hypertension in human, but not mice.

1.2.2.3 Residues surrounding renin cleavage site

AngI (amino acid position at 1-10 of AGT) is highly conserved between species; however, amino acid residues adjacent to AngI is not conserved in species (Fig. 1.3). Therefore, N-terminal amino sequences of AGT has been assumed as the factor regulating the interaction of AGT to renin and catalytic activity of renin cleavage of AGT. It was reported that removal of tyrosine at C-terminal position 2 of renin cleavage site abolished synthetic substrate activity to hog renin [102]. Tewksbury et al. showed the potential effect of residue at position 3 at C-terminal side of renin cleavage site on $K_{cat}/K_m$ of renin [103]. Cumin et al. showed that N-terminal residues at position 11 to 14 play an important role of renin cleavage efficiency, especially 12’ Ile replaced by Tyr in human tetradecapeptide [104]. Replacement of residues at position 1 and 2 adjacent to renin cleavage site of human AGT to mimic the two residues at the same position of canine AGT significantly enhanced $K_{cat}/K_m$ of canine renin [105]. Moreover, amino acids at position 12 and 13 of human N-terminus of AGT are seemed to affect the catalytic reaction of renin [106]. Although these show the important roles of amino acid residues adjacent to renin cleavage site in catalytic efficiency of renin and AngI production, these studies used synthetic peptides and in vitro
assays. Therefore, in vivo evidence, such as mouse study, is needed to investigate functions of amino acids around renin cleavage site in AGT metabolism.

Figure 1.3 Amino acid sequence of N-terminus part of natural renin substrates.

1.2.2.4 Conserved residues in des(AngI)AGT structure

AGT is cleaved by renin to release 10 amino acids-long AngI and des(AngI)AGT which is the remaining part of AGT (98% of AGT). AngI is cleaved by ACE to produce AngII, which affects and regulates many physiological and pathophysiological functions. However, studies of functions of remaining part of protein, des(AngI)AGT is limited. It has been reported that des(AngI)AGT inhibited angiogenesis [107, 108]. Lu et al. reported that repopulation of des(AngI)AGT in hepatocyte-specific AGT deficient mice increased body weight and liver steatosis, but did not affect atherosclerosis, showing that des(AngI)AGT exerted an AngII-independent functions [59]. Interestingly, Lu et al. aligned the amino acid sequences of AGT from human, rat, mouse and zebrafish, and mapped the sequences onto the published structure of AGT [99], and found two highly conserved regions, the β-sheet and loop region, on the surface of AGT which are distal to
renin-interacting face (Fig. 1.4). These regions may display renin or AngII-independent functions.

![Diagram showing renin-interacting face and conserved regions in des(AngI)AGT]

**Figure 1.4** Structural analysis of highly conserved regions in des(AngI)AGT

<table>
<thead>
<tr>
<th>Region</th>
<th>Residues</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-face</td>
<td>253, 274, 422</td>
<td>K, H, E</td>
</tr>
<tr>
<td>Loop</td>
<td>291-301</td>
<td>FWVDNISVSV</td>
</tr>
</tbody>
</table>

1.3 **Regulation of AGT expression**

AGT production is regulated by many molecules, such as steroid hormones, cytokines and AngII [109]. These molecules regulate the activation of transcription factors which stimulate AGT transcription and consequently enhance AGT synthesis. There are many other factors affecting AGT expression as well, such as nutritional conditions [110], sodium concentration [111-113], hypoxia [114], fatty acids [115], insulin [116] and hyperglycemia [117].
Glucocorticoid (GC) administration increased AGT expression and production [118-121]. Two glucocorticoid response elements (GREs) in 5’-flanking region of AGT gene are essential for GC induction [122, 123]. Estrogen is one of steroid hormones inducing AGT expression. Estrogen administration increased AGT mRNA level in a tissue-specific manner in ovariectomized rats [124]. It has been found that a half-palindromic estrogen-response element is located on 5’-flanking region of rat AGT gene [123], showing a mechanism how estrogen activates AGT transcription.

In addition, AGT transcription can be induced by cytokines, such as interleukin-1α (IL-1α) and tumor necrosis factor (TNF), which is mediated by an acute phase response element (APRE) [125-127]. Ron et al. indicated that glucocorticoid is necessary for IL-1α-induced AGT expression [128]. Lipopolysaccharide (LPS), a bacterial endotoxin stimulating host immune defense, induces AGT production [129], which is mediated by TNF-a and IL-6 pathways [130, 131], showing important effects of inflammation on AGT gene activation.

Positive feedback regulation of AGT synthesis mediated by AngII, its end product, is one of important regulations of AGT production [132]. For examples, 3 days of AngII infusion significantly increased plasma AGT concentration in male Sprague-Dawley rats with elevated plasma and renal AGT mRNA abundance [133].

1.4 Tissue and cellular distribution of AGT

AGT is expressed in many tissues, such as liver, kidney, brain, adipose tissue, aorta and heart [119]. Local AGT expression may regulate local tissue functions. AGT is mainly produced in hepatocytes in liver [134]. Liver-derived AGT is released to circulation [56,
Deficiency of hepatocyte-derived AGT significantly decreased plasma and renal AGT concentration, showing liver is the primary source of AGT production. Moreover, AGT has been reported to express in kidney [136] and primarily in S3 segment of renal proximal tubules [137-139]. Diet-feeding with different salt levels [112] or plasma glucose concentrations [140, 141] regulates renal AGT expression. Adipose tissues have been reported as one of sources of plasma AGT [142-144]. AGT expression in adipose tissues [145-147] is associated with development of obesity [148, 149] and affects blood pressure [143, 144]. AGT mRNA is also found in whole aorta as well, predominantly in adventitia [150, 151]. Tissue-specific AGT expression is associated with regulation of local or systemic RAS.

1.5 Conclusions and perspectives

Evidence showing the critical roles of AGT in regulation of the RAS and RAS-related diseases in a tissue-dependent and dose-dependent manners. Although it is known that AGT determines the production of angiotensin peptides which affect physiological and pathological functions, the studies investigating the functions of whole and cleaved AGT in AGT metabolism and consequently angiotensin peptide-associated functions are limited. There are still many unknown questions: (1) There are several in vitro studies showing the potential effects of amino acids surrounding renin cleavage site on influencing renin cleavage of AGT. However, there is no in vivo study to prove the effects of these amino acids of AGT on renin activity. Do these amino acids regulate angiotensin peptides production and consequently affect its pathophysiological functions in vivo? (2) Highly
conserved regions of AGT were reported in our previous publication. The functions of these conserved regions remain unclear. Do these highly conserved regions of AGT determine its interaction with megalin, an AGT receptor expressing in renal proximal tubule? Do these highly conserved regions of AGT influence AngII production and atherosclerosis? Therefore, to address these questions, I aim to investigate the roles of specific amino acids of AGT in AGT metabolism locally and systemically as well as consequently AngII-mediated functions in this study.

1.6 Specific aims

To address some of the fundamental questions discussed above, I propose two specific aims:

**Aim 1:** To investigate the role of two amino acids next to the renin cleavage site of AGT in blood pressure and atherosclerosis (Chapter three).

**Aim 2:** To determine the effects of highly conserved regions of AGT on blood pressure and atherosclerosis. (Chapter four).

<table>
<thead>
<tr>
<th>Table 1-1 Nonstandard Abbreviations and Acronyms</th>
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<tbody>
<tr>
<td><strong>Abbreviation</strong></td>
</tr>
<tr>
<td>RAS</td>
</tr>
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<td>Renin angiotensin system</td>
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<tr>
<td>AGT</td>
</tr>
<tr>
<td>Angiotensinogen</td>
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<tr>
<td>AngI</td>
</tr>
<tr>
<td>Angiotensin I</td>
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<tr>
<td>AngII</td>
</tr>
<tr>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ACE</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AT1 receptor</td>
</tr>
<tr>
<td>Angiotensin II receptor type 1</td>
</tr>
<tr>
<td>hepAGT&lt;sup&gt;+/+&lt;/sup&gt; mice</td>
</tr>
<tr>
<td>Hepatocyte-specific AGT&lt;sup&gt;+/+&lt;/sup&gt; mice</td>
</tr>
<tr>
<td>hepAGT&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
</tr>
<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>siRNAs</td>
</tr>
<tr>
<td>AAV</td>
</tr>
<tr>
<td>Null AAV</td>
</tr>
<tr>
<td>L11V;Y12I</td>
</tr>
<tr>
<td>SPR</td>
</tr>
<tr>
<td>WT.AAV</td>
</tr>
<tr>
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<tr>
<td>W292A.AAV</td>
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<td>loop.AAV</td>
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<td>loop deletion.AAV</td>
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<td>β-sheet and loop.AAV</td>
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</table>
CHAPTER 2. GENERAL METHODS

This chapter describes the methods, including mouse blood collection, plasma total cholesterol measurement, plasma AGT measurement, systolic blood pressure measurement, quantification of atherosclerotic lesion sizes and statistical analysis, used in studies in Chapter 3 and Chapter 4.

2.1 Materials

Reagents used in studies in Chapter 3 and 4 are listed below.

<p>| Table 2-1 Reagents and chemicals used in studies in Chapter 3 and 4. |</p>
<table>
<thead>
<tr>
<th>Reagents</th>
<th>Vendor or Source</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol E for plasma total cholesterol concentrations</td>
<td>Wako Chemicals USA</td>
<td>999-02601</td>
</tr>
<tr>
<td>Mouse total angiotensinogen (AGT) ELISA</td>
<td>IBL America</td>
<td>27413</td>
</tr>
<tr>
<td>Human total angiotensinogen (AGT) ELISA</td>
<td>IBL America</td>
<td>27412</td>
</tr>
<tr>
<td>Plasma and renal AngII concentrations by liquid chromatography–mass spectrometry</td>
<td>Attoquant Diagnostics</td>
<td></td>
</tr>
<tr>
<td>Mouse renin ELISA kit</td>
<td>R&amp;D Systems</td>
<td>DY4277</td>
</tr>
<tr>
<td>DuoSet Ancillary Reagent Kit 2</td>
<td>R&amp;D Systems</td>
<td>DY008</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>Bachem</td>
<td>H-1705</td>
</tr>
</tbody>
</table>
2.2 Plasma profiles

Mouse blood were collected in the presence of EDTA (final concentration: 1.8 mg/ml) and a proteinase inhibitor cocktail (provided by Attoquant Diagnostics GmbH, Vienna, Austria) to prevent degradation of AGT and angiotensin peptides. During the experiment, blood was collected through retro-orbital bleeding at selected intervals. Cardiac bleeding via right ventricle was used to collect blood at termination.

Plasma total cholesterol concentrations were measured using an enzymatic commercial kit (Cat # 999-02601; Wako Chemicals USA).

Plasma AGT concentrations were measured using a mouse AGT ELISA kit (Code # 27413; IBL America) or a human AGT ELISA kit (Code # 27412; IBL America).

Plasma renin concentrations were measured using an ELISA kit (Mouse renin ELISA kit, Cat # DY4277; R&D Systems).

2.3 Systolic blood pressure measurement

Systolic blood pressure was measured on conscious mice using a non-invasive tail-cuff system (Coda 8, Kent Scientific Corporation) following our standard protocol [152]. Data were collected and analyzed based on 20 measurements of each mouse every day for 3 consecutive days. Mean systolic blood pressure of each mouse from the 3-day measurements was used for data analysis.
2.4 Quantification of atherosclerosis

An *en face* method was used to measure atherosclerotic lesions on the intimal surface of the aorta following the recent AHA statement [153], as detailed in our standard protocol posted on protocols.io (dx.doi.org/10.17504/protocols.io.bfy8jpwz) and reported in our publications (a few examples,[57, 59, 154, 155]). Briefly, the aorta was dissected and fixed in 10% neutrally buffered formalin overnight. The adventitial tissues were removed, then the intimal surface was exposed by a longitudinal cut and pinned on a black wax surface. Images of *en face* aortas were taken by a Nikon digital camera (Nikon digital sight DS-Ri1) with a mm ruler for calibration. Lesions were traced manually in the region from the junction of the myocardium (after the aortic root) to 3 mm distal from the left subclavian artery using Nikon NIS-Elements software (NIS-Elements AR 5.11.00.) under a dissecting microscope. One individual measured the lesion areas, and a senior staff member who did not know the animal groups verified the measurements independently. Lesion size was presented as percent lesion area as below:

\[
\text{Percent lesion area (\%)} = \frac{\text{Atherosclerotic lesion (mm}^2\text{)}}{\text{Intimal area of the aorta (mm}^2\text{)}} \times 100
\]

2.5 Statistical analysis

Data are represented as means ± standard error of means (SEM). SigmaPlot version 14.0 (SYSTAT Software Inc.) was used for statistical analyses. To compare multiple-group data, one-way ANOVA followed by Holm-Sidak method was used for normally distributed variables that passed equal variance test. Kruskal-Wallis one-way ANOVA on
Ranks followed by Dunn’s method was used for data that did not pass either normality or equal variance test. \( P < 0.05 \) was considered statistically significant.
CHAPTER 3. ROLE OF AMINO ACIDS ADJACENT TO RENIN CLEAVAGE SITE IN ANGIOTENSINOGEN IN ANGIOTENSIN II-MEDIATED FUNCTIONS IN MICE

This chapter is based on our published article titled “The Two Amino Acids Proximate to the Renin Cleavage Site in Angiotensinogen Do Not Affect Angiotensin II-mediated Functions”, with Chia-Hua Wu as the first author (Arterioscler Thromb Vasc Biol. 2020 Jul 9;ATVBAHA120314048. doi: 10.1161/ATVBAHA.120.314048.)

3.1 Abstract

Renin cleavage of angiotensinogen (AGT) has species specificity. Since the residues at positions 11 and 12 are different between human AGT and mouse AGT, we determined whether these two residues in AGT affect renin cleavage and angiotensin II-mediated blood pressure regulation and atherosclerosis using an adeno-associated viral (AAV) approach for manipulating AGT in vivo. Hepatocyte-specific AGT deficient (hepAGT−/−) mice in an LDL receptor deficient background were infected with AAVs containing a null insert, human AGT, or mouse AGT expressing the same residues of the human protein at positions 11 and 12 [mouse AGT (L11V;Y12I)]. Expression of human AGT in hepAGT−/− mice led to high plasma human AGT concentrations without changes in plasma endogenous mouse AGT, plasma renin concentrations, blood pressure, or atherosclerosis. This is consistent with human AGT not being cleaved by mouse renin. To determine whether the residues at positions 11 and 12 in human AGT lead to the inability of mouse renin to cleave human AGT, hepAGT−/− mice were injected with AAV encoding mouse AGT (L11V;Y12I). Expression of mouse AGT (L11V;Y12I) resulted in increased plasma mouse AGT concentrations, reduced renin concentrations, and increased renal
AngII concentrations that were comparable to their concentrations in hepAGT+/+ mice. This mouse AGT variant increased blood pressure and atherosclerosis in hepAGT−/− mice to the magnitude of hepAGT+/+ mice. In conclusion, replacement of L11 and Y12 to V11 and I12, respectively, in mouse AGT does not affect renin cleavage, blood pressure, and atherosclerosis in LDL receptor deficient mice.

3.2 Introduction

Renin cleavage of angiotensinogen (AGT) is the rate-limiting step in producing bioactive angiotensin (Ang) peptides [61, 156]. The product of AGT and renin interaction is AngI, the first 10 amino acids of the amino terminus of secreted AGT. AngI is highly conserved from fly, rodents, to human [61]. One study reported that a conserved disulfide bond between Cys18 and Cys138 in human AGT (Cys18 and Cys137 in mouse AGT) regulated cleavage of human AGT by human renin in vitro [99]. However, our in vivo study demonstrated that this conserved disulfide bond in AGT did not affect AngII production and AngII-mediated functions [56], implicating potentially different results from in vitro versus in vivo conditions.

Renin cleavage of AGT displays species specificity [157-159]. For example, human AGT is not cleaved by mouse renin, and vice versa as demonstrated in transgenic mouse models [158, 159]. Similarly, human AGT is not cleaved by canine renin, but replacement of the residues at positions 11 and 12 of human AGT to the two residues in canine AGT enhanced its cleavage by canine renin in an in vitro study [105], implicating that these two residues adjacent to the renin cleavage site are important for regulating AGT cleavage by
renin. However, it has not been determined whether the two amino acids adjacent to the AngI sequence in AGT affect renin cleavage and AngII-mediated functions in vivo.

In this study, we used an adeno-associated viral vector (AAV)-driven expression approach to either populate the full length of human AGT or mouse AGT that its two residues at positions 11 and 12 were replaced with the two residues in the human protein. This approach provided a rapid and efficient manipulation of AGT to study amino acid determinants of its interaction with renin in vivo and the consequent AngII-mediated physiological and pathophysiological functions such as such as blood pressure and atherosclerosis.

3.3 Methods and materials

3.3.1 Animals

Development of hepatocyte-specific AGT deficient mice has been reported previously [160-162]. AGT floxed (termed “Agt \(^{f/f}\) x albumin-Cre\(^{-/-}\) (hepAGT\(^{+/+}\)) and Agt \(^{f/f}\) x albumin-Cre\(^{-/-}\) (hepAGT\(^{-/-}\)) littermates were used for experiments described in this manuscript. Genotypes were determined prior to weaning and validated after termination by Cre PCR and confirmed by measuring plasma AGT concentrations during the study and after termination. Male and female mice in a low-density lipoprotein receptor (LDLR)\(^{-/-}\) background were studied separately following the recent ATVB Council statement [163]. All animal experiments reported in this manuscript were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee (IACUC protocol number 2006-0009 or 2018-2968).
3.3.2 Production and injection of adeno-associated viral (AAV) vector

AAV vectors (serotype 2/8) driven by a hepatocyte-specific thyroxine-binding globulin (TBG) promoter were produced by the Vector Core in the Gene Therapy Program at the University of Pennsylvania. Three AAV vectors were made: (1) a null insertion (null AAV; used as control), (2) encoding the human AGT (human AGT.AAV), and (3) encoding the mouse AGT with L11V;Y12I mutations (L11V;Y12I.AAV). The L11V;Y12I represented that the N-terminal leucine at position 11 and tyrosine at position 12 in mouse AGT were replaced by valine and isoleucine, respectively, to mimic the two amino acids at the same positions in human AGT (Figure 3.1A).

**Figure 3.1 Experimental designs of AGT mutation and mouse experiment.**

(A) N-terminal amino acids 1 - 10 (AngI) are the same between human and mouse AGT. Amino acids at positions 11 and 12 are different between human and mouse AGT. Mouse (L11V;Y12I) represent the N-terminal leucine at position 11 and tyrosine at position 12 in mouse AGT replaced by valine and isoleucine, respectively, to mimic the two amino acids at the same positions of human AGT. (B) Schematic summary of the experimental design: LDL receptor -/- mice were injected intraperitoneally with AAVs encoding a null insert, human AGT, or mouse AGT (L11V;Y12I). Two weeks after AAV injections, mice were fed a Western diet for 12 weeks. Blood pressure was measured on week 10 during the Western diet feeding, and atherosclerosis was measured after termination.
All mice were fed a normal laboratory diet (Diet # 2918; Envigo), and were injected intraperitoneally with AAV vectors (3 x 10^{10} genome copies/mouse). hepAGT^{+/+} mice were injected with null AAV as a positive control at 8 - 12 weeks of age. Sex- and age-matched hepAGT^{-/-} mice were randomized to receive AAVs containing a null insert (negative control), human AGT, or L11V;Y12I mouse AGT. Two weeks after injection, to induce hypercholesterolemia, all study mice have been fed a Western diet (Diet # TD.88137; Envigo) for 12 weeks (Figure 3.1B). This diet has high saturated fat, modest cholesterol enrichment, and no cholate. The diet contents of this Western diet are shown in the Table 3-1.

Table 3-1 The diet contents of Western diet

<table>
<thead>
<tr>
<th>Contents</th>
<th>% by weight</th>
<th>% kcal</th>
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</thead>
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<tr>
<td>Protein</td>
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<td>15.2</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>48.5</td>
<td>42.7</td>
</tr>
<tr>
<td>Fat</td>
<td>21.2</td>
<td>42.0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

3.3.3 Osmotic Mini Pump Implantation and Angiotensin II Infusion

Saline or angiotensin II (AngII 0.5 µg/kg/min; Cat# H-1706; Bachem) was infused subcutaneously via mini osmotic pumps (Alzet Model # 2006; Durect Corp.) as described in our publication. 5 Male hepAGT^{-/-} mice were infused with either saline or AngII, and their hepAGT^{+/+} littermates were infused with saline. Mice were sedated with isoflurane and pumps were implanted subcutaneously on the right flank of each mouse. Incisions were closed with surgical staples and a topical anesthetic cream (LMX4; Ferndale Laboratories) was applied immediately after surgery to relieve pain.
3.3.4 Plasma profiles

Methods of blood collection, and measurements of plasma total cholesterol and plasma AGT were described in Chapter 2 General methods.

Plasma renin concentrations in male mice were measured using a radioimmunoassay method: 5 Mouse plasma samples (8 μl) were incubated in an assay buffer (Na2HPO4 0.1 M, EDTA 0.02 M, maleate buffer pH 6.5, phenylmethyl-sulfonyl fluoride 2 μl; total volume of 250 μl) with an excess of rat AGT at 37°C for 30 min. The reaction was terminated by placing samples at 100°C for 5 min. AngI generated in each sample was quantified by radioimmunoassay using a commercially available kit (Cat # 1553; DiaSorin).

Plasma renin concentrations in female mice were measured using an ELISA kit (Mouse renin ELISA kit, Cat # DY4277; R&D Systems). We compared plasma renin data between male and female hepAGT+/+ and -/- mice side-by-side using this ELISA kit as shown in Figure 3.2.

![Figure 3.2 Plasma renin concentrations in hepAGT+/+ and hepAGT-/- male and female mice measured using a mouse renin ELISA kit.](image_url)
3.3.5 Systolic blood pressure measurements

Systolic blood pressure measurement was described in Chapter 2 General methods.

3.3.6 Quantification of atherosclerosis

An *en face* method was described in Chapter 2 General methods.

3.3.7 Angiotensin II (AngII) Measurements

Plasma and renal AngII concentrations were measured using liquid chromatography–mass spectrometry by Attoquant Diagnostics GmbH (Vienna, Austria). All samples were assayed in a blinded manner.

3.3.8 Statistical analysis

Statistical analysis was described in Chapter 2 General methods.

3.4 Results

3.4.1 Population of human AGT in hepAGT*−/−* mice did not affect systolic blood pressure and atherosclerosis

In both hepAGT*+/+* and *−/−* male mice infected with null AAV, human AGT was not detected in mouse plasma by ELISA. In hepAGT*−/−* mice infected with AAV encoding human AGT, plasma human AGT concentrations were comparable to plasma AGT concentrations of humans [164] (Figure 3.3A), but did not affect plasma concentrations of endogenous mouse AGT (Figure 3.3B), demonstrating induction of human AGT did not affect endogenous mouse AGT concentrations in hepAGT*−/−* mice. Plasma renin
concentrations were comparable between hepAGT−/− mice infected with null AAV and human AGT.AAV, both of which were higher than those in hepAGT+/+ mice injected with null AAV (Figure 3.3C). Plasma total cholesterol concentrations were above 1,000 mg/dl after 12 weeks of Western diet feeding with no differences among the 3 groups (Figure 3.3D). Since AngII, the major effector peptide of the renin-angiotensin system, regulates blood pressure and contributes to development of atherosclerosis, we measured these two parameters. Expression of human AGT in hepAGT−/− mice did not change systolic blood pressure and atherosclerotic lesion size, when compared to hepAGT−/− mice infected with null AAV (Figure 3.3E and F). However, systolic blood pressure and atherosclerotic lesion size were much higher in hepAGT+/+ mice than in hepAGT−/− mice injected with either null AAV or human AGT AAV. These findings were also confirmed in female mice (Figure 3.4), demonstrating that human AGT does not affect AngII-mediated functions in mice irrespective of sex.
Figure 3.3 Human AGT does not affect blood pressure and atherosclerosis in male hepAGT⁻/⁻ mice.

All study mice were in an LDL receptor⁻/⁻ background. (A) Plasma human AGT concentrations were measured by an ELISA kit that was specific for human AGT. *P<0.05 versus the other 2 groups by Kruskal–Wallis one-way ANOVA on Ranks with Dunn’s method. (B) Plasma mouse AGT concentrations were measured by an ELISA kit that was specifically targeting mouse AGT. *P<0.05 versus hepAGT⁺/+ mice injected with null AAV by Kruskal–Wallis one-way ANOVA on Ranks with Dunn’s method. (C) Plasma renin concentrations were measured by radioimmunoassay. *P<0.05 versus hepAGT⁺/+ mice injected with null AAV by Kruskal–Wallis one-way ANOVA on Ranks with Dunn’s method.
method. (D) Plasma total cholesterol concentrations were measured by an enzymatic assay kit and analyzed with one-way ANOVA. P>0.05. (E) Systolic blood pressure was measured using a tail-cuff system 3 weeks after AAV injection. *P<0.05 versus hepAGT+/+ mice injected with null AAV by one-way ANOVA with Holm-Sidak method. (F) Atherosclerotic lesion area was quantified by an en face method. *P<0.05 versus hepAGT+/+ mice injected with null AAV by one-way ANOVA with Holm-Sidak method. N = 16 - 22/group. hepAGT+/+ mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 18 - 24 weeks old at termination.
3.4.2 Expression of mouse AGT L11V;Y12I in hepAGT−/− mice normalized AngII production and blood pressure, and augmented atherosclerosis

To determine whether N-terminal residues at positions 11 and 12 of AGT affect renin cleavage, AAV encoding mouse AGT with the L11V and Y12I substitutions were
injected into male hepAGT<sup>−/−</sup> mice with an LDL receptor<sup>−/−</sup> background. Administration of mouse AGT(L11V;Y12I).AAV led to increased plasma mouse AGT concentrations to be comparable as in hepAGT<sup>+/+</sup> mice injected with null AAV (Figure 3.5A). Plasma renin concentrations were not different between hepAGT<sup>+/+</sup> mice and hepAGT<sup>−/−</sup> mice populated with mouse AGT(L11V;Y12I), but lower than hepAGT<sup>−/−</sup> mice infected with null AAV (Figure 3.5B). To confirm that increased plasma renin concentrations in hepAGT<sup>−/−</sup> were due to lower AngII production, we infused either saline or AngII to male hepAGT<sup>−/−</sup> mice and compared with their male hepAGT<sup>+/+</sup> littermates infused with saline. Plasma renin concentrations were much higher in hepAGT<sup>−/−</sup> mice infused with saline, compared with their wild type littermates. Infusion of AngII in hepAGT<sup>−/−</sup> mice suppressed plasma renin concentrations to the comparable concentrations as in hepAGT<sup>+/+</sup> mice (Figure 3.6).

As reported previously [57], plasma AngII concentrations were not different between hepAGT<sup>+/+</sup> and hepAGT<sup>−/−</sup> mice, which were also not affected by infection of AAVs encoding mouse AGT(L11V;Y12I) (Figure 3.5C). Renal AngII concentrations were higher in hepAGT<sup>+/+</sup> mice and hepAGT<sup>−/−</sup> mice infected with mouse AGT(L11V;Y12I).AAV than in hepAGT<sup>−/−</sup> mice infected with null AAV (Figure 3.5D). Plasma total cholesterol concentrations were not different among the 3 groups (Figure 3.5E). Population of mouse AGT(L11V;Y12I) in hepAGT<sup>−/−</sup> mice led to increased systolic blood pressure (Figure 3.5F) and atherosclerotic lesion size (Figure 3.5G), which were comparable to the two phenotypes in hepAGT<sup>+/+</sup> mice infected with null AAV. A separate study demonstrated that female hepAGT<sup>−/−</sup> mice with an LDL receptor<sup>−/−</sup> background populated with mouse AGT(L11V;Y12I) had comparable blood pressure and
atherosclerotic lesion size as female hepAGT*+/ mice injected with null AAV (Figure 3.7), while their plasma cholesterol concentrations were not different (Figure 3.8).

Therefore, the amino-terminal amino acids at positions 11 and 12 of human AGT that are adjacent to the renin cleavage site do not determine renal AngII production, blood pressure, and atherosclerosis in mice irrespective of sex.
Figure 3.5 Mouse AGT(L11V;Y12I) increases blood pressure and augmented atherosclerosis in male hepAGT+/− mice.
All study mice were in an LDL receptor−/− background. (A) Plasma mouse AGT concentrations were measured by ELISA. *P<0.05 versus the other 2 groups by Kruskal–
Wallis one-way ANOVA on Ranks with Dunn’s method. (B) Plasma renin concentrations were measured by radioimmunoassay. *P<0.05 versus the other 2 groups by Kruskal–Wallis one-way ANOVA on Ranks with Dunn’s method. AngII concentrations in plasma (C) and kidney (D) were measured using LC-MS/MS. *P<0.05 by Kruskal–Wallis one-way ANOVA on Ranks with Dunn’s method. (E) Plasma total cholesterol concentrations were measured using an enzymatic kit and analyzed by one-way ANOVA. (F) Systolic blood pressure was measured using a tail-cuff system. *P<0.05 versus the other 2 groups by one-way ANOVA with Holm-Sidak method. (G) Atherosclerotic lesion area was quantified by en face method. *P<0.05 versus the other 2 groups by one-way ANOVA with Holm-Sidak method. N = 10 - 13/group. hepAGT+/+ mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 18 – 26 weeks old at termination.

![Graph showing plasma renin concentrations](image)

**Figure 3.6 Infusion of AngII suppresses plasma renin concentrations in male hepAGT−/− mice.**

All study mice were in an LDL receptor−/− background. Plasma renin concentrations were measured by ELISA. *P<0.05 by Kruskal-Wallis one-way ANOVA with Dunn's Method. N = 6 - 7/group. hepAGT+/+ mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 20 – 24 weeks old at termination.
Figure 3.7 Mouse AGT(L11V;Y12I) increases blood pressure and augmented atherosclerosis in female hepAGT−/− mice.

All study mice were in an LDL receptor−/− background. (A) Plasma mouse AGT concentrations were measured by ELISA. *P<0.05 by Kruskal-Wallis one-way ANOVA on Ranks with Dunn’s method. (B) Plasma renin concentrations were measured by ELISA. *P<0.001 by one-way ANOVA with Holm-Sidak method. (C) Systolic blood pressure was measured using a tail-cuff system. *P<0.05 by one-way ANOVA with Holm-Sidak method. (D) Atherosclerotic lesion area was quantified by an en face method. *P<0.05 by one-way ANOVA with Holm-Sidak method. N = 5 - 11/group. hepAGT+/+ mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 22 – 31 weeks old at termination.
Figure 3.8 Mouse AGT(L11V;Y12I) does not change plasma cholesterol concentrations in female hepAGT<sup>−/−</sup> mice.

All study mice were in an LDL receptor<sup>−/−</sup> background. Plasma total cholesterol concentrations were measured using an enzymatic kit and analyzed by one-way ANOVA. N = 5 - 11/group. hepAGT<sup>+/+</sup> mice: hepatocyte-specific AGT<sup>+/−</sup> mice; hepAGT<sup>−/−</sup> mice: hepatocyte-specific AGT deficient mice. hepAGT<sup>+/+</sup> and hepAGT<sup>−/−</sup> study mice were littermates. Mice were 22 – 31 weeks old at termination.

3.5 Discussion

Using an AAV approach for manipulating AGT in hepatocytes, this study reports two novel findings. First, human AGT does not affect AngII-mediated blood pressure regulation and atherosclerosis in LDL receptor<sup>−/−</sup> mice lacking endogenous AGT in hepatocytes. Second, replacement of the amino-terminal amino acids at positions 11 and 12 of mouse AGT to mimic human AGT does not impair renin cleavage of AGT, resulting in normal renal AngII production and AngII-mediated blood pressure regulation and atherosclerosis.
Previous studies using human AGT transgenic mice have demonstrated that the presence of human AGT in mice does not affect systolic blood pressure [165-167]. In human AGT transgenic mice, human AGT was expressed in mice with normal concentrations of endogenous mouse AGT and relatively low mouse renin concentrations, which does not rule out the possibility that lack of cleavage of human AGT is due to low mouse renin concentrations. Our mouse model has several advantages: (1) We used a mouse model having low endogenous mouse AGT concentrations but high mouse renin concentrations. The low plasma concentrations of mouse AGT were sufficient to enable normal growth and kidney development, but led to much lower blood pressure, compared to their wild type littermates [56, 57, 59]. Therefore, this mouse model is relevant to physiological regulation of the renin-angiotensin system. More importantly, our mouse model can address the caveats raised above in previous studies. (2) Our mouse model was on an LDL receptor−/− background, so we were able to study two different AngII-induced physiologic and pathophysiologic functions, namely blood pressure and atherosclerosis. (3) The AAV vector encoding human AGT with a hepatocyte-specific promoter led to human AGT produced in hepatocytes, mimicking the normal processing of AGT production in liver and secretion to the circulation [57, 135].

In hepAGT−/− mice infected with AAV containing human AGT, plasma human AGT concentrations were above 10 μg/ml, which were 3-4-fold higher than plasma endogenous mouse AGT concentrations in wild type mice. Although mouse plasma renin concentrations were greatly elevated in hepAGT−/− mice compared with wild type mice due to removal of AngII-mediated negative feedback, the presence of human AGT did not correct plasma renin concentrations, elevate blood pressure, or contribute to the
development of atherosclerosis. Therefore, expression of human AGT did not restore AngII-mediated functions in hepAGT<sup>−/−</sup> mice, which supports the notion that human AGT is not cleaved by mouse renin in vivo [157-159, 165]. These results confirm previous findings of species specificity of AGT and renin interaction [157, 158, 168], and extend these findings to the in vivo physiological and pathophysiological setting.

In contrast to population of human AGT in hepAGT<sup>−/−</sup> mice, population of mouse AGT with L11 and Y12 being substituted to the residues of human AGT reduced plasma renin concentrations and increased renal AngII concentrations to those of wild type mice. As anticipated with these findings, the responses of blood pressure and atherosclerotic lesion size were restored in hepAGT<sup>−/−</sup> mice infected with murine AGT containing these two residues of human AGT. These findings provide compelling evidence that the two amino acid substitutions between murine and human do not affect renin cleavage of AGT and the subsequent AngII-mediated blood pressure regulation and atherosclerosis in hepAGT<sup>−/−</sup> mice. Our results do not support a role for these two residues of human AGT as determinants of the species-specificity of the reaction between AGT and renin.

Consistent with our previous report [57], plasma AngII concentrations were not different between hepAGT<sup>+/+</sup> and <sup>−/−</sup> mice, irrespective of infection with AAV containing a null insert or mutated AGT. In contrast, population of mouse AGT with L11V;Y12I substitutions increased renal AngII concentrations to levels of hepAGT<sup>+/+</sup> mice, consistent with changes of blood pressure and atherosclerosis in these mice. In this study, AngII concentrations in kidney were considerably higher than in plasma. AT1a receptor is abundant in kidney [169]. Indeed, previous studies have reported that expression of AT1a
receptor in kidney, but not in the vasculature, contributes to blood pressure regulation by AngII [170, 171]. Since renal AngII, but not plasma AngII, reflects AngII effects on blood pressure regulation and atherosclerosis, these results suggest that local tissue AngII (e.g. renal) is important in these effects of the renin-angiotensin system.

Plasma AGT concentrations in male hepAGT−/− mice injected AAV encoding mouse AGT(L11V;Y12I) were compared to those in their hepAGT+/+ littermates. We noted much lower plasma AGT concentrations in female hepAGT−/− mice injected AAV encoding mouse AGT(L11V;Y12I) than in their hepAGT+/+ littermates. This sex difference has also been reported in mice injected with AAV encoding PCSK9 [172, 173]. The mechanism responsible for this sex difference is unclear. Despite the lower plasma AGT concentrations in female mice, mouse AGT(L11V;Y12I), but not human AGT, increased both blood pressure and atherosclerosis in female hepAGT−/− mice to those of their wild type littermates. These results support that the amino-terminal amino acids at positions 11 and 12 of human AGT that are adjacent to the renin cleavage site do not determine renal AngII production, blood pressure, and atherosclerosis in mice independent of sex.

Infusion of AngII in hepAGT−/− mice suppressed plasma renin concentrations, supporting that lower blood pressure and atherosclerosis in hepatocyte-specific deletion of AGT are attributed to lower AngII concentrations. In future studies, we will determine whether blockade of AT1 receptors would inhibit the increased blood pressure and atherosclerosis in hepAGT−/− mice injected with AAVs encoding mouse AGT.
(L11V,Y12I), which would further support that AngII and AT1 receptor interaction is the mechanism to blood pressure regulation and atherosclerosis in these mice.

Given the species-specificity of AGT cleavage by renin, we hypothesize that differences of either other amino acid sequences or the tertiary protein structure of AGT affect the renin cleavage. We therefore aligned AGT protein sequences from different species and mapped highly conserved regions of surface residues onto the structure of the AGT:renin complex that was published by Zhou et al. [99]. One conserved region of AGT is anticipated to be in direct contact with renin and therefore expected to influence species specific interaction between AGT and renin. Future studies will aim to manipulate non-conserved residues in this renin-interacting region to determine whether certain amino acid sequences in this region affect the tertiary protein structure or interaction with renin.
CHAPTER 4. ROLES OF CONSERVED SEQUENCES IN THE β-SHEET AND LOOP REGIONS OF ANGIOTENSINOGEN IN ANGIOTENSIN II-MEDIATED FUNCTIONS IN MICE

This chapter is based on a manuscript in preparation, titled “Conserved sequences in the β-sheet and loop regions of angiotensinogen synergistically regulate plasma angiotensinogen production and affect angiotensin II-mediated functions”, with Chia-Hua Wu as the first author. Figures based on experiments conducted by others are listed here: figure 4.1A was made by Dr. Craig Vander Kooi and data of figure 4.1B was provided by Dr. Dudley Strickland.

4.1 Abstract

Angiotensinogen (AGT) is the only precursor of all angiotensin peptides. Our previous publication shows that two highly conserved regions, the β-sheet and loop region on the face of AGT distal to renin interacting face might have renin-independent functions. In this study, we determined whether these highly conserved regions in AGT affect angiotensin II-mediated blood pressure regulation and atherosclerosis using an adeno-associated viral (AAV) approach for manipulating AGT in vivo. Surface plasmon resonance analysis showed that mutation of W292 in AGT loop region decreased its binding affinity to megalin, a protein that is abundant in renal proximal convoluted tubules. To determine whether mutation of W292A affected AngII-mediated functions in mice, we used hepatocyte-specific AGT deficient (hepAGT−/−) mice in an LDL receptor deficient background were infected with AAVs containing a null insert, WT AGT, or mouse AGT with W292A mutation. Repopulation of this mutated AGT increased plasma AGT concentrations, blood pressure, and atherosclerosis, to the magnitudes, which were
comparable to those in hepAGT^{+/-} mice. Subsequently, to determine whether triple nucleotide mutations in the \(\beta\)-sheet or the loop region of AGT affect AngII-mediated functions. Infection of AAVs encoding mutations K253A;H274A;E422A of the \(\beta\)-sheet region or W297A;S303A;V304A of the loop region in hepAGT^{-/-} mice increased plasma AGT concentrations, blood pressure, and atherosclerosis to be equivalent as in hepAGT^{+/-} mice, supporting that the 3 conserved residues of each region do not affect AGT metabolism and AngII-mediated functions. Next, we replaced the residues of 291-301 in the loop region with a GA linker, and infected hepAGT^{-/-} mice with AAVs encoding this protein. Expression of this mutated AGT moderately increased plasma AGT concentrations, but increased blood pressure and atherosclerosis to a level comparable to hepAGT^{+/-} mice. We then determined whether conserved sequences in the \(\beta\)-sheet and loop regions would have synergistic effects on AngII-mediated functions. Administration of AAV encoding mutated sequences in both the \(\beta\)-sheet region (K253A;H274A;E422A) and the loop region (W297A;S303A;V304A) did not increase plasma AGT concentrations, blood pressure, or atherosclerosis in hepAGT^{-/-} mice, supporting that the conserved sequences of these two regions have synergistic effects on AGT metabolism and function. In conclusion, the conserved sequences in the \(\beta\)-sheet and loop regions of AGT synergistically regulate AGT protein metabolism and AngII-mediated functions.

4.2 Introduction

Angiotensinogen (AGT) is the only precursor of the renin angiotensin system (RAS), which plays critical roles in physiological and pathological functions, such as regulations of sodium/water homeostasis and blood pressure [1, 2]. AGT is a member of
the serine-proteinase inhibitor (serpin) family, but it does not have the enzyme activity [174, 175]. Intact mouse AGT without a signal peptide has 453 amino acids. The N-terminal 10-residues peptide, angiotensin I (Ang I), is cleaved by renin. The remaining cleaved AGT protein, termed des(AngI)AGT, has 98% of the parent protein, which biological functions remain unclear [61].

AGT is a key factor affecting AngII-mediated functions, including blood pressure and atherosclerosis [56, 57, 59]. However, Lu et al. reported that repopulation of des(AngI)AGT in hepatocyte-specific AGT knockout mice which have deficiency of plasma AGT did not regulate AngII-mediated functions, such as blood pressure and atherosclerosis. Interestingly, repopulation of des(AngI)AGT increased body weight gain, liver weight and liver triglyceride content, showing other biological functions of des(AngI)AGT [59].

Analysis of conservation in AGT shows several potential functional surface regions in des(AngI)AGT by comparing the amino acid sequences in human, rat, mouse and zebrafish and mapping the sequences onto the protein structure of AGT [59, 99]. The N-terminal AngI, important for production of other angiotensin peptides, is highly conserved in species. There are other three unknown surface conserved regions having observed. Interestingly, based on the region interacting with renin, one conserved region directly interacts with renin and two other conserved regions, the β-sheet face (K253, H274, and E422) and the loop region (residues 291-301) with strictly conserved hydrophobic residues W292 and V299 along with S298, on the distal face of AGT [59]. Conserved regions of AGT distal to renin-binding region might have renin-independent functions, such as the interaction with other proteins, instead of interaction with renin.
AGT is primarily synthesized in liver and delivered to kidney [134, 135]. The uptake of AGT in kidney is mediated by megalin, a multiligand endocytic receptor abundant in renal proximal tubules and regulates the homeostasis of the RAS in the kidney [57, 138]. It has been found that renal AngII concentration is associated with the development of atherosclerosis [56, 57, 59, 156], showing the important roles of the interaction of AGT with megalin in AngII-mediated functions. How the interaction of AGT with megalin is regulated is still unclear. We hypothesize that conserved regions of AGT distal to renin-binding region determine its interaction with megalin and further affect AngII-mediated functions. Therefore, in this study, we made several mutations on the β-sheet face and loop region of AGT and repopulated mutated AGT by adeno-associated virous expression approach in hepatocyte-specific AGT deficient mice to investigate the effects of conserved regions of AGT on AGT metabolism and AngII-mediated functions.

4.3 Methods and materials

4.3.1 Surface plasmon resonance (SPR)

Surface plasmon resonance was performed by Dr. Dudley Strickland at the University of Maryland. Briefly, binding of AGT (GenScript) to megalin was assessed using a Biacore 3000 optical biosensor system (GE Healthcare Life Sciences). Full length megalin was coupled to a Sensor Chip CM5 (BR-1003-99; GE Healthcare Life Sciences). Various concentrations of AGT ligand in HBS-P Buffer supplemented with 1 mM Ca2+ was flowed over the surface of the megalin-coupled sensor chip at a rate of 20 µL/min at 25°C.
4.3.2 Animals

Development of hepatocyte-specific AGT deficient mice has been reported previously [56, 57, 59]. AGT floxed (termed “Agt^f/f^”) x albumin-Cre^- (hepAGT^+/+) and Agt^f/f^ x albumin-Cre^+/+ (hepAGT^-/-) littermates were used for experiments described in this manuscript. Genotypes were determined by Cre PCR and confirmed by measuring plasma AGT concentrations. Male and female mice in an LDL receptor^-/- background were studied separately following the recent ATVB Council statement [163]. All animal experiments reported in this manuscript were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee (IACUC protocol number 2006-0009 or 2018-2968).

4.3.3 Production and injection of adeno-associated viral (AAV) vector

AAV vectors (serotype 2/8) driven by a hepatocyte-specific thyroxine-binding globulin (TBG) promoter were produced by the Vector Core in the Gene Therapy Program at the University of Pennsylvania. Seven AAV vectors were made: (1) a null insertion (null AAV; used as control), (2) encoding the mouse wild type AGT (WT.AAV), (3) encoding the mouse AGT with the β-sheet mutations at K253A, H274A, E422A (β-sheet.AAV), (4) encoding the mouse AGT with the loop region mutation at W292A (W292A.AAV), (5) encoding the mouse AGT with the loop region mutations at W292A, S298A, V299A (loop.AAV), (6) encoding the mouse AGT with the loop region mutations at whole conserved sequences which were replaced by a GA linker (loop deletion.AAV), and (7) encoding the mouse AGT with the β-sheet and loop region mutations at K253A, H274A, E422A.
E422A, W292A, S298A, and V299A (β-sheet and loop.AAV) (Fig. 4.1A). The W292A represents the replacement of N-terminal tryptophan at position 292 to alanine in mouse AGT. The triple mutation in the loop region (W292A;S298A;V299A) represents that tryptophan at position 292, serine at position 298 and valine at position 299 were replaced by alanine, respectively in mouse AGT. The loop deletion represents that an amino acid sequence from tryptophan 292 to valine 299 was replaced by a GA linker, which stabilized the protein structure. The triple mutation in the β-sheet (K253A;H274A;E422A) represents that lysine at position 253, histidine at position 274 and glutamic acid at 422 were replaced by alanine, respectively in mouse AGT. The double mutations in the β-sheet and loop region (K253A;H274A;E422A;W292A;S298A;V299A) represent that the combination of the triple mutations in the β-sheet and the triple mutations in the loop region in mouse AGT.

All mice were fed a normal laboratory diet (Diet # 2918; Envigo), and were injected intraperitoneally with AAV vectors (3 x 10^{10} genome copies/mouse). hepAGT^{+/+} mice were injected with null AAV as a positive control at 8 - 12 weeks of age. Sex- and age-matched hepAGT^{-/-} mice were randomized to receive AAVs containing a null insert (negative control), WT AGT, mutated mouse AGT with the β-sheet, mutated mouse AGT with W292A, mutated mouse AGT with the loop mutation, mutated mouse AGT with loop deletion mutation, or mutated mouse AGT with the β-sheet and loop mutations. Two weeks after AAV injection, to induce hypercholesterolemia, all study mice have been fed a Western diet (Envigo, Diet # TD.88137) for 12 weeks. The diet contents of this Western diet are shown in the Table 3-1.
4.3.4 Plasma profiles

Methods of blood collection, and measurements of plasma total cholesterol, plasma AGT and plasma renin were described in Chapter 2 General methods.

4.3.5 Systolic blood pressure measurements

Systolic blood pressure measurement was described in Chapter 2 General methods.

4.3.6 Quantification of atherosclerosis

An en face method was described in Chapter 2 General methods.

4.3.7 Body composition

Body composition, including whole body fat mass, lean tissue mass, free water, and total body water, was measured on conscious mice with EchoMRI-100™ (Echo Medical Systems, Houston, TX, USA).

4.3.8 RNA isolation and quantitative PCR

Total RNA in liver was extracted with a commercial kit (Cat # AS1280;Promega) configured with an automated Maxwell 16 purification system (Promega). To quantitate mRNA abundance, total RNA was reversely transcribed with an iScript™ cDNA Synthesis Kit (Cat #170-8891; Bio-Rad, Hercules, CA, USA), and quantitative PCR (qPCR) was performed to quantify mRNA abundance using an SsoFast™ EvaGreen® Supermix kit (Cat # 172-5204; Bio-Rad) on a Bio-Rad CFX96 cycler. Data were analyzed using ∆∆Ct method and normalized by a geometric mean of internal controls including α-
actin, GAPDH and RPLP2. Primers used for AGT are 5’-CTGACCCAGTTTTGCCAC and 5’-AACCTCTCATCGTTCTTGG.

4.3.9 Statistical analysis

Statistical analysis was described in Chapter 2 General methods.

4.4 Results

4.4.1 The loop region of AGT might determine the interaction with megalin

The interaction of AGT and megalin predisposes to atherosclerosis [57]. To investigate whether the loop region of AGT determines the interaction with megalin, we analyzed the protein-protein interaction by surface plasmon resonance (SPR). A single nucleotide mutation W292A on the loop region of AGT decreased the binding affinity of AGT to megalin (Fig. 4.1B), suggesting that the loop region of AGT affects its binding to megalin.
Figure 4.1 Single nucleotide mutation on the loop region (W292A) decreases the binding affinity of AGT to megalin.

(A) Structural analysis of highly conserved regions in des(Ang I) AGT based on bioinformatic analyses of AGT gene sequences from human, rat, mouse, and zebrafish. Four highly conserved surface regions (Core, renin interacting, β-sheet face, and loop) were identified in des(AngI) AGT domain. (B) Protein-protein interactions between AGT and megalin analyzed by SPR.
4.4.2 W292 in the loop region does not affect AGT metabolism and AngII-mediated functions.

Then, we determined whether W292A mutation in the loop region of AGT affects AngII-mediated functions. Expression of AGT with W292A (Fig. 4.2A) significantly increased plasma AGT concentration in hepAGT<sup>−/−</sup> mice (Fig. 4.2B), showing that W292A of AGT does not affect AGT expression and synthesis. Plasma renin concentration acts as an indirect indicator of plasma AngII concentration because of negative feedback regulation of AngII production. HepAGT<sup>−/−</sup> mice repopulated with W292 AGT significantly decreased plasma renin concentration which level was comparable with hepAGT<sup>+/+</sup> mice injected with null AAV and hepAGT<sup>−/−</sup> infected with WT.AAV (Fig. 4.2C). Mice body weights were measured weekly to monitor their general health. HepAGT<sup>−/−</sup> mice have less body weight and fat mass gains during 12-weeks western diet feeding (Fig. 4.3 A and B), which is consistent to our previous findings [59]. HepAGT<sup>−/−</sup> infected with W292A AGT did not show significant difference in weekly body weight and fat mass gains compared to hepAGT<sup>+/+</sup> mice injected with null AAV and hepAGT<sup>−/−</sup> injected with WT.AAV (Fig. 4.3A and B). There was no significant difference in plasma total cholesterol concentrations among groups (Fig. 4.3C). Since blood pressure and development of atherosclerosis are regulated by AngII, the major bioactive peptide of the classical renin-angiotensin system, we measured these two parameters to investigate whether mutated AGT affects blood pressure and atherosclerosis. HepAGT<sup>−/−</sup> mice expressing W292A AGT displayed significantly higher systolic blood pressure and atherosclerotic lesion size than those in hepAGT<sup>−/−</sup> mice injected with null AAV. Moreover, hepAGT<sup>−/−</sup> mice infected with this mutated AGT did not show significantly
change in systolic blood pressure and atherosclerotic lesion size, compared to hepAGT+/+ mice infected with null AAV and hepAGT−/− mice infected with WT.AAV (Fig. 4.2D and E), demonstrating that AGT with W292A mutation does not affect AngII-mediated functions in mice.

Figure 4.2 Single nucleotide mutation (W292) on the loop region does not affect AngII-mediated functions in male hepAGT−/− mice. All study mice were in an LDL receptor−/− background. (A) Nucleotide mutation on the loop region (B) Plasma mouse AGT concentrations were measured by a mouse AGT ELISA kit.
*P<0.05 by Kruskal–Wallis one-way ANOVA on Ranks with Dunn’s method. (C) Plasma renin concentrations were measured by radioimmunoassay. *P<0.05 by Kruskal-Wallis one-way ANOVA on Ranks with Dunn’s method. (D) Systolic blood pressure was measured using a tail-cuff system 9 weeks after AAV injection. *P<0.05 by one-way ANOVA with Holm-Sidak method. (E) Atherosclerotic lesion area was quantified by an en face method. *P<0.05 by one-way ANOVA with Holm-Sidak method. N = 5 - 8/group. hepAGT+/+ mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 22 – 31 weeks old at termination. Mice were 22 – 28 weeks old at termination.

Figure 4.3 W292A on the loop region in AGT does not affect body weight, lean and fat mass as well as plasma total cholesterol in male hepAGT−/− mice. All study mice were in an LDL receptor−/− background. (A) Weekly body weights in 16 weeks. (B) Lean mass and fat mass gains after 12 weeks western diet feeding. (C) Plasma total cholesterol concentrations were measured by an enzymatic assay kit and analyzed with one-way ANOVA. P>0.05. N = 5 - 8/group. hepAGT+/+ mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 22 – 28 weeks old at termination.
4.4.3 Three nucleotides in the β-sheet and loop regions, respectively, do not affect AGT metabolism and AngII-mediated functions.

In hepAGT<sup>−/−</sup> mice infected with AAV encoding mutated AGT with triple nucleotide mutations (K253A;H274A;E422A) on the β-sheet region (Fig. 4.4A), plasma AGT concentrations were comparable to plasma AGT concentrations in hepAGT<sup>+/+</sup> mice infected with null AAV and hepAGT<sup>+/−</sup> infected with WT AGT.AAV (Fig. 4.4B), demonstrating that triple nucleotide mutations on the β-sheet did not affect AGT production. Plasma renin concentrations were comparable between hepAGT<sup>−/−</sup> mice infected with β-sheet.AAV and hepAGT<sup>+/−</sup> mice injected with null AAV, both of which were lower than those in hepAGT<sup>−/−</sup> mice injected with null AAV (Fig. 4.4C). Repopulation of mutated AGT with the β-sheet mutation did not affect weekly body weight and fat mass gains, compared to hepAGT<sup>+/−</sup> mice injected with null AAV and hepAGT<sup>−/−</sup> mice infected with WT.AAV (Fig. 4.5A and B). There was no significant difference in plasma total cholesterol concentrations among groups (Fig. 4.5C). Expression of AGT with the β-sheet mutation in hepAGT<sup>−/−</sup> mice had increased systolic blood pressure and atherosclerotic lesion sizes which were comparable to hepAGT<sup>+/−</sup> mice infected with null AAV and hepAGT<sup>−/−</sup> mice infected with WT.AAV (Fig. 4.4D and E), demonstrating that AGT with the β-sheet mutation does not affect AngII-mediated functions in mice.
Figure 4.4 Triple nucleotide mutations on the β-sheet in AGT do not affect AngII-mediated functions in male hepAGT+/− mice. All study mice were in an LDL receptor−/− background. (A) Nucleotide mutations on the β-sheet (B) Plasma mouse AGT concentrations were measured by a mouse AGT ELISA kit. *P<0.05 by one-way ANOVA with Holm-Sidak method. (C) Plasma renin concentrations were measured by radioimmunoassay. *P<0.05 by Kruskal-Wallis one-way ANOVA on Ranks with Dunn’s method. (D) Systolic blood pressure was measured using a tail-cuff system 9 weeks after AAV injection. *P<0.05 by one-way ANOVA with Holm-Sidak method. (E) Atherosclerotic lesion area was quantified by an en-face method. *P<0.05 by one-way ANOVA with Holm-Sidak method. N = 3 - 6/group. hepAGT+/− mice:
hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 18 – 32 weeks old at termination.

Figure 4.5 Triple nucleotide mutations on the β-sheet in AGT do not affect body weight, plasma total cholesterol as well as lean and fat mass in male hepAGT−/− mice. All study mice were in an LDL receptor −/− background. (A) Weekly body weights in 12 weeks. (B) Lean mass and fat mass gains after 12 weeks western diet feeding. (C) Plasma total cholesterol concentrations were measured by an enzymatic assay kit and analyzed with one-way ANOVA. P>0.05. N = 3 - 6/group. hepAGT+/+ mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 18 – 32 weeks old at termination.
We investigated whether three nucleotide mutations in the loop region (W292A, S298A, V299A) of AGT (Fig. 4.6A) affect AGT production and AngII-mediated functions. Expression of AGT with triple nucleotide mutations on the loop region did not significantly change plasma AGT concentrations, compared to plasma AGT concentrations in hepAGT+/+ mice infected with null AAV and hepAGT−/− infected with WT AGT.AAV (Fig. 4.6B). Plasma renin concentrations were comparable between hepAGT−/− mice infected with loop.AAV, hepAGT−/− mice infected with WT AGT.AAV and hepAGT+/+ mice, all of which were lower than those in hepAGT−/− mice injected with null AAV (Fig. 4.6C). HepAGT−/− mice infected with this mutated AGT did not show significant difference in body weight and fat mass gains compared to WT controls (Fig. 4.7A and B). Also, plasma total cholesterol concentrations of hepAGT−/− mice infected with loop.AAV were not significantly different from WT controls, although they were higher than those in hepAGT−/− injected with null AAV (Fig. 4.7C). Repopulation of this mutated AGT did not significantly change systolic blood pressure and atherosclerosis, compared to hepAGT+/+ mice infected with null AAV and hepAGT−/− infected with WT AGT.AAV (Fig. 4.6D and E). Taken together, three residues in the β-sheet and loop region, respectively, are not critical for AGT metabolism and consequently AngII-mediated functions.
Figure 4.6 Triple nucleotide mutations on the loop region in AGT do not affect AngII-mediated functions in male hepAGT-/- mice.
All study mice were in an LDL receptor-/- background. (A) Nucleotide mutations on the loop region (B) Plasma mouse AGT concentrations were measured by a mouse AGT ELISA kit. *P<0.05 by one-way ANOVA with Holm-Sidak method. (C) Plasma renin concentrations were measured by radioimmunoassay. *P<0.05 by Kruskal-Wallis one-way ANOVA on Ranks with Dunn’s method. (D) Systolic blood pressure was measured using a tail-cuff system 9 weeks after AAV injection. *P<0.05 by one-way ANOVA with Holm-Sidak method. (E) Atherosclerotic lesion area was quantified by an en face method. *P<0.05 by one-way ANOVA with Holm-Sidak method. N = 7 - 10/group. hepAGT+/+
mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 20 – 34 weeks old at termination.

Figure 4.7 Triple nucleotide mutations on the loop region do not affect body weight, plasma total cholesterol as well as lean and fat mass in male hepAGT−/− mice. All study mice were in an LDL receptor−/− background. (A) Weekly body weights in 12 weeks. (B) Lean mass and fat mass gains after 12 weeks western diet feeding. *P<0.05 by one-way ANOVA with Holm-Sidak method. (C) Plasma total cholesterol concentrations were measured by an enzymatic assay kit. *P<0.05 by one-way ANOVA with Holm-Sidak method. N = 7 - 10/group. hepAGT+/+ mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 20 – 34 weeks old at termination.
4.4.4 Expression of mutated AGT with loop deletion in hepAGT\(^{-/-}\) mice moderately affects AGT production but does not affect AngII-mediated functions.

We then investigated whether replacement of the residues of 291-301 in the loop region with a GA linker (Fig. 4.8A) affects AGT metabolism and AngII-mediated functions in hepAGT\(^{-/-}\) mice infected with loop deletion.AAV. HepAGT\(^{-/-}\) mice infected with loop deletion AAV moderately increased plasma AGT concentrations, compared to hepAGT\(^{+/+}\) mice injected with null AAV (Fig. 4.8B), showing that the whole conserved sequence replacement in the loop region moderately affects AGT production. Liver mRNA abundance of AGT in hepAGT\(^{-/-}\) infected with loop deletion.AAV was not significantly different from hepAGT\(^{+/+}\) mice injected with null AAV (Fig. 4.9A), suggesting that this mutation does not affect expression of AGT. In hepAGT\(^{-/-}\) mice infected with AAVs encoding this protein, plasma renin concentration (131.4 ± 18.1 ng/ml) significantly increased compared to hepAGT\(^{+/+}\) injected with null AAV (39.3 ± 3.7 ng/ml) (Fig. 4.8C). Although there was no significant difference in plasma renin concentration between hepAGT\(^{-/-}\) mice infected with loop deletion.AAV and hepAGT\(^{-/-}\) mice injected with null AAV (482.9 ± 154.9 ng/ml), plasma renin concentrations of hepAGT\(^{-/-}\) mice infected with loop deletion.AAV were about 3.5 times lower than plasma renin concentrations of hepAGT\(^{-/-}\) mice injected with null AAV (Fig. 4.8C), suggesting that systemic AngII concentrations were higher in hepAGT\(^{-/-}\) mice infected with loop deletion.AAV than in hepAGT\(^{-/-}\) mice injected with null AAV. HepAGT\(^{-/-}\) mice infected with this mutated AGT did not show significant difference in body weight and fat mass gains compared to WT controls (Fig. 4.9B and C). Plasma total cholesterol concentrations were not significantly different among groups (Fig. 4.9D). For AngII-mediated functions, expression of AGT
with the loop deletion mutation still significantly increased systolic blood pressure and atherosclerosis compared to hepAGT\textsuperscript{+/+} mice injected with null AAV (Fig. 4.8D and E). A separate female mice study demonstrated that female hepAGT\textsuperscript{-/-} mice with an LDL receptor\textsuperscript{-/-} background populated with mutated AGT with loop deletion had comparable blood pressure and atherosclerotic lesion size as female hepAGT\textsuperscript{+/+} mice injected with null AAV, while their body weight and fat mass gains did not change (Fig. 4.10). In summary, replacement of the whole conserved sequences of the loop region with a GA linker does not affect blood pressure and atherosclerosis.
Figure 4.8 GA linker substitution on the loop region in AGT decreases plasma AGT concentration but did not affect AngII-mediated functions in male hepAGT−/− mice. All study mice were in an LDL receptor−/− background. (A) Nucleotide mutation on the loop region (B) Plasma mouse AGT concentrations were measured by a mouse AGT ELISA kit. *P<0.05 by Kruskal–Wallis one-way ANOVA on Ranks with Dunn’s method. (C) Plasma renin concentrations were measured by radioimmunoassay. *P<0.05 by Kruskal-Wallis one-way ANOVA on Ranks with Dunn’s method. (D) Systolic blood pressure was measured using a tail-cuff system 9 weeks after AAV injection. *P<0.05 by one-way ANOVA with Holm-Sidak method. (E) Atherosclerotic lesion area was quantified by an en face method. *P<0.05 by one-way ANOVA with Holm-Sidak method. N = 6 - 7/group.
hepAGT<sup>+/+</sup> mice: hepatocyte-specific AGT<sup>+/+</sup> mice; hepAGT<sup>−/−</sup> mice: hepatocyte-specific AGT deficient mice. hepAGT<sup>+/+</sup> and hepAGT<sup>−/−</sup> study mice were littermates. Mice were 18 – 20 weeks old at termination.

Figure 4.9 GA linker substitution on the loop region in AGT does not affect mRNA abundance, body weight, lean and fat mass as well as plasma total cholesterol in male hepAGT<sup>−/−</sup> mice. All study mice were in an LDL receptor<sup>−/−</sup> background. (A) mRNA abundance of liver AGT. *P<0.05 by Kruskal–Wallis one-way ANOVA on Ranks with Dunn’s method. (B) Weekly body weights in 12 weeks. (C) Lean mass and fat mass gains after 12 weeks western diet feeding. *P<0.05 by one-way ANOVA with Holm-Sidak method. (D) Plasma total cholesterol concentrations were measured by an enzymatic assay kit and analyzed with one-way ANOVA. P>0.05. N = 6 - 7/group. hepAGT<sup>+/+</sup> mice: hepatocyte-specific AGT<sup>+/+</sup> mice; hepAGT<sup>−/−</sup> mice: hepatocyte-specific AGT deficient mice. hepAGT<sup>+/+</sup> and hepAGT<sup>−/−</sup> study mice were littermates. Mice were 18 – 20 weeks old at termination.
Figure 4.10 GA linker substitution on the loop region in AGT did not affect growth and AngII-mediated functions in female hepAGT−/− mice. All study mice were in an LDL receptor−/− background. (A) Weekly body weights in 12 weeks. (B) Lean mass and fat mass gains after 12 weeks western diet feeding. *P<0.05 by Kruskal-Wallis one-way ANOVA on Ranks with Dunn’s method. (C) Plasma total cholesterol concentrations were measured by an enzymatic assay kit and analyzed with one-way ANOVA. P>0.05. (D) Systolic blood pressure was measured using a tail-cuff
system 9 weeks after AAV injection. *P<0.05 by one-way ANOVA with Holm-Sidak method. (E) Atherosclerotic lesion area was quantified by an en face method. *P<0.05 by one-way ANOVA with Holm-Sidak method. N = 6 - 10/group. hepAGT+/+ mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 18 – 28 weeks old at termination.

4.4.5 Repopulation of AGT with mutations on the β-sheet and loop region in hepAGT−/− mice affects AGT production and AngII-mediated functions.

We then determined whether conserved sequences in the β-sheet and loop regions would have synergistic effects on AngII-mediated functions. Administration of AAV encoding mutated sequences in both the β-sheet region (K253A;H274A;E422A) and the loop region (W297A;S303A;V304A) (Fig. 4.11A) did not increase plasma AGT concentrations, compared to hepAGT−/− mice injected with null AAV (Fig. 4.11B). Although mRNA abundance of liver AGT in hepAGT−/− mice infected with β-sheet and loop.AAV was significantly lower than hepAGT+/+ mice injected with null AAV, it was higher than hepAGT−/− mice injected with null AAV (Fig. 4.12A), suggesting that the β-sheet and loop region mutation might affect AGT synthesis in liver. Plasma renin concentrations of hepAGT−/− mice infected with β-sheet and loop.AAV significantly increased compared to WT controls (Fig. 4.11C). Body weight and fat mass gains were decreased (Fig. 4.12B and C). Plasma total cholesterol concentrations were not significantly different among groups (Fig. 4.12D). Administration of AGT with the β-sheet and loop region mutation did not increase systolic blood pressure and atherosclerosis compared to hepAGT−/− mice injected with null AAV (Fig. 4.11D and E). A separate female mice study demonstrated that female hepAGT−/− mice with an LDL receptor−/− background populated with AGT with mutations on the β-sheet and loop region had
significantly decreased blood pressure and atherosclerotic lesion size as female hepAGT-/- mice injected with null AAV, while their body weight and fat mass gains also decreased (Fig. 4.13). In summary, the β-sheet and loop region synergistically regulate AGT metabolism and then consequently AngII-mediated functions in both male and female mice.
Figure 4.11 Mutations on the β-sheet (K253A, H274A, E422A) and loop region (W292A, S298A, V299A) of AGT decrease plasma AGT concentration and affect AngII-mediated functions in male hepAGT−/− mice. All study mice were in an LDL receptor−/− background. (A) Nucleotide mutations on the β-sheet and loop region (B) Plasma mouse AGT concentrations were measured by a mouse AGT ELISA kit. *P<0.05 by Kruskal–Wallis one-way ANOVA on Ranks with Dunn’s method. (C) Plasma renin concentrations were measured by radioimmunoassay. *P<0.05 by Kruskal–Wallis one-way ANOVA on Ranks with Dunn’s method. (D) Systolic blood
pressure was measured using a tail-cuff system 9 weeks after AAV injection. *P<0.05 by Kruskal-Wallis one-way ANOVA on Ranks with Dunn’s method. (E) Atherosclerotic lesion area was quantified by an en face method. *P<0.05 by one-way ANOVA with Holm-Sidak method. N = 7 - 10/group. hepAGT+/+ mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 18 – 20 weeks old at termination.

![Image](attachment:image.png)

**Figure 4.12** Mutations on the β-sheet (K253A, H274A, E422A) and loop region (W292A, S298A, V299A) of AGT do not affect body weight, plasma total cholesterol as well as lean and fat mass in male hepAGT−/− mice.

All study mice were in an LDL receptor−/− background. (A) mRNA abundance of liver AGT. *P<0.05 by Kruskal-Wallis one-way ANOVA on Ranks with Dunn’s method. (B) Weekly body weights in 12 weeks. (C) Lean mass and fat mass gains after 12 weeks western diet feeding. *P<0.05 by one-way ANOVA with Holm-Sidak method. (D) Plasma total cholesterol concentrations were measured by an enzymatic assay kit and analyzed with one-way ANOVA. P>0.05. N = 7 - 10/group. hepAGT+/+ mice: hepatocyte-specific
AGT<sup>+/+</sup> mice; hepAGT<sup>−/−</sup> mice: hepatocyte-specific AGT deficient mice. hepAGT<sup>+/+</sup> and hepAGT<sup>−/−</sup> study mice were littermates. Mice were 18 – 20 weeks old at termination.

Figure 4.13 Mutations on the β-sheet (K253A, H274A, E422A) and loop region (W292A, S298A, V299A) of AGT decrease plasma AGT concentration and affect AngII-mediated functions in female hepAGT<sup>−/−</sup> mice. All study mice were in an LDL receptor<sup>−/−</sup> background. (A) Plasma mouse AGT concentrations were measured by a mouse AGT ELISA kit. *P<0.05 by Kruskal–Wallis
one-way ANOVA on Ranks with Dunn’s method. (B) Plasma renin concentrations were measured by radioimmunoassay. *P<0.05 by Kruskal-Wallis one-way ANOVA on Ranks with Dunn’s method. (C) Weekly body weights in 12 weeks. (D) Lean mass and fat mass gains after 12 weeks western diet feeding. (E) Systolic blood pressure was measured using a tail-cuff system 9 weeks after AAV injection. *P<0.05 by Kruskal-Wallis one-way ANOVA on Ranks with Dunn’s method. (F) Atherosclerotic lesion area was quantified by an en face method. *P<0.05 by one-way ANOVA with Holm-Sidak method. N = 9 - 11/group. hepAGT+/+ mice: hepatocyte-specific AGT+/+ mice; hepAGT−/− mice: hepatocyte-specific AGT deficient mice. hepAGT+/+ and hepAGT−/− study mice were littermates. Mice were 19 – 31 weeks old at termination.

4.5 Discussion

This study reports three novel findings. First, the loop region might determine the interaction of AGT with megalin. Secondly, mutation on each β-sheet or loop region of AGT does not affect AGT function and AngII-mediated functions. Thirdly, the β-sheet and loop region of AGT synergistically regulate AGT synthesis and consequently AngII-mediated functions.

The β-sheet and loop region, highly conserved amino acids on the surface of AGT which are distal to renin interacting face, were firstly reported in our previous publication [59]. Lu et al. reported that these conserved regions in des(AngI)AGT might have important roles in the regulation of AGT functions. Moreover, our previous publication demonstrates the critical roles of AGT and megalin in renal RAS homeostasis and atherosclerosis [57]. Therefore, in this study, firstly, we tested the protein-protein interaction of W292A AGT and megalin. We found that the loop region of AGT affected its binding to megalin (Fig. 4.1B). However, expression of W292A AGT in hepAGT−/− mice did not affect systolic blood pressure and atherosclerosis (Fig. 4.2). We further investigated the effects of the β-sheet and loop region on AGT metabolism and AnII-
mediated functions by several different mutations. Triple nucleotide mutations on the β-sheet did not affect AGT production and blood pressure as well as atherosclerosis (Fig. 4.4), indicating that the β-sheet is not critical for regulation of AGT synthesis. However, the β-sheet synergistically regulated AGT production and consequently AngII-mediated functions with the loop region (Fig. 4.11). Triple nucleotide mutations on the loop region did not affect AGT synthesis and AngII-mediated functions (Fig. 4.6). Expression of replacement of whole conserved sequence of the loop region with a GA linker in hepAGT−/− mice showed the reduced plasma AGT concentration without changing blood pressure or atherosclerosis, compared to hepAGT+/+ mice injected with null AAV or hepAGT−/− mice infected with WT.AAV (Fig. 4.8), implicating that the loop region of AGT is involved in the regulation of AGT synthesis. Interestingly, combination of triple nucleotide mutations on both β-sheet and loop region showed the most profound effects on AGT synthesis and abolished AngII-mediated functions (Fig. 4.11). Studies of functions of des(AngI)AGT are limited. Des(AngI)AGT has been reported to inhibit angiogenesis [107, 176]. Des(AngI)AGT can form high molecule AGT by RCL(reactive center loop)-β-sheet polymerization [177], which is associated with pre-eclampsia [178, 179]. These studies reveal the other important roles of des(AngI)AGT in pathophysiological functions. In this study, we demonstrate that highly conserved sequences, the β-sheet and loop region of AGT regulate AGT functions and the loop region affect the binding of AGT to megalin.

Lu et al. demonstrated that des(AngI)AGT, a conserved serpin domain of AGT, exert an AngII-independent functions, such as body weight gain, liver weight, and liver triglyceride content [59]. How des(AngI)AGT increases body weight gain, liver
triglyceride content and liver weight remain unclear. In this study, we observed that mutations on these highly conserved sequences did not change fat mass or body weight gain compared to WT controls, showing that the β-sheet or the loop region are not essential for the regulation of body weight and fat mass gains in mice fed a Western diet.

It has been shown that leukocytes-specific components of the RAS are not critical for atherosclerosis, although leukocytes are involved in the pathogenesis of atherosclerosis. For example, deficiency of AGT [59] or AT1 receptor [18] in bone marrow-derived cells did not affect atherosclerosis. Moreover, AngII concentration and AT1 receptor expression are predominant in kidney, revealing the potential pivotal roles of renal RAS in atherosclerosis. AGT and megalin interactions contribute to renal RAS homeostasis and atherosclerosis. In this study, we investigated how AGT and megalin interaction regulates AngII-mediated functions. However, there are some difficulties limiting the explores of interactions of megalin with highly conserved sequences of AGT: (1) big size of megalin (about 600 kD)[180, 181] causing the difficulty of purification, and (2) low AGT protein production due to mutations. Moreover, since mutation on the β-sheet did not change plasma AGT concentration and blood pressure as well as atherosclerosis, we only investigated whether the loop region is essential for AGT binding to megalin in SPR analysis. The data showed that W292 in the loop region is involved in the interaction with megalin. Megalin has four ligand-binding regions [180]. Therefore, future studies will aim to study potential interactions of AGT with which ligand-binding domains to investigate and explore the potential roles of local RAS in development of atherosclerosis.
CHAPTER 5. GENERAL DISCUSSION – LIMITATIONS, SOLUTIONS AND CONTRIBUTIONS OF STUDIES REGARDING SPECIFIC DOMAINS OF AGT IN ANGII-MEDIATED FUNCTIONS

This chapter is an overall discussion based on chapter 3 and 4. This chapter discusses the limitations of these studies, the ways to solve problems, future directions, and importance and contributions of these studies in cardiovascular diseases.

5.1 Amino acids adjacent to renin cleavage site (Chapter 3)

Our results showed that two amino acids at position 11 and 12 adjacent to renin cleavage site in mouse AGT do not affect renin cleavage of AGT and consequently AngII-mediated functions in hepAGT−/− mice. This results support the in vitro finding of Hatae et al. [182], but are contrary to the data of Burton et al who demonstrated that the amino acids are critical for renin cleavage of AGT by using synthetic tetradecapeptides and human or canine renin. These results implicate that amino acid residues next to renin cleavage site in different species might have different effects on catalytic activity of renin from different species. In addition to amino acids, tertiary protein structure of AGT and specificities of renin from different species might also contribute to the efficiency of renin cleavage of AGT.

N-terminal amino acids around renin cleavage sites also regulate the catalytic activity of renin. 12’ isoleucine replaced by tyrosine residue in human tetradecapeptide significantly decreased $k_{cat}$ of renin [104]. Six residues surrounding the renin cleavage site of human AGT are important for human renin action [106]. In a preeclamptic patient, there was a mutation with the replacement of leucine by phenylalanine at position 10 of AGT (L10F), which altered the reaction of AGT with renin [183]. These studies show the other
potential amino acids regulating renin activity. Our previous studies reported that there are highly conserved regions in AGT, including hydrophobic core, AngI, renin interacting face, β-sheet and loop region [59]. Conserved sequence of renin interacting face may determine the efficiency of renin cleavage of AGT. Future studies will aim to manipulate conserved residues in this renin-interacting region to determine whether certain amino acid sequences in this region affect the tertiary protein structure or interaction with renin.

In addition, plasma L11V;Y12I mouse AGT repopulation showed difference in hepAGT−/− male (Fig. 3.5A) and female mice (Fig. 3.7A). Much lower plasma AGT concentrations was expressed in female hepAGT−/− mice infected AAV encoding mouse AGT(L11V;Y12I) than in their hepAGT+/+ littermates. This sex difference has also been reported in mice injected with AAV encoding PCSK9 [172, 173], although the mechanism of AAV-mediated protein expression contributing to sex difference is unclear. For mouse AGT measurement, we have been using the same ELISA kit for more than 10 years (the best kit among 4 commercially available kits we tested) and documented the batch number of the ELISA kit in each experiment as a lab routine. Despite the same kit which has been used, different batch number and measurements conducted by different persons might get the slightly different absolute values of AGT concentrations (Table 5-1).

Table 5-1 Plasma AGT concentrations in wild type mice measured using different batches of the mouse AGT ELISA kit (Code # 27413; IBL America).

<table>
<thead>
<tr>
<th>Batch #</th>
<th>Date performed</th>
<th>Sex</th>
<th>Plasma AGT (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1K-226</td>
<td>11/07/2013</td>
<td>Male</td>
<td>2868 ± 668</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>3063 ± 299</td>
</tr>
<tr>
<td>1I-412</td>
<td>02/03/2015</td>
<td>Male</td>
<td>3592 ± 253</td>
</tr>
<tr>
<td>1J-427</td>
<td>10/05/2017</td>
<td>Male</td>
<td>3620 ± 128</td>
</tr>
<tr>
<td>1F-713</td>
<td>03/08/2018</td>
<td>Female</td>
<td>1798 ± 30</td>
</tr>
<tr>
<td>1C-3</td>
<td>09/04/2018</td>
<td>Male</td>
<td>1760 ± 56</td>
</tr>
<tr>
<td>1B-820</td>
<td>05/30/2020</td>
<td>Male</td>
<td>1554 ± 47</td>
</tr>
</tbody>
</table>
Systolic blood pressures in hepAGT+/+ mice were about 140-160 mmHg in Human AGT AAV study and L11V;Y12I AGT AAV study (Chapter 3) and conserved regions AGT studies (Chapter 4) by using a tail-cuff system, CODA high throughput system (Kent Scientific). Hypertension is defined as a systolic blood pressure (SBP) ≥130 mmHg and a diastolic blood pressure (DBP) ≥80 mmHg in adults [184]. Compared to human, SBP in hepAGT+/+ mouse was relatively high. The accuracy of this tail-cuff system is affected by many factors, including environment temperature, stress on mice and tail blood volume status. (1) Multiple training sessions, (2) taping the distal part of the tail on the platform, (3) cover the animal holder to reduce light and noise, and (4) check the temperature of platform are helpful to minimize agitation of mice and reduce issues in SBP measurement [185, 186]. In contrast, a tail-cuff blood pressure measurement has several advantages. For example, measurements in conscious animals are close to physiological conditions. A tail-cuff system is a simple and rapid way to test blood pressure within multiple animals, without anesthesia or invasive surgery. Moreover, in our previous publications, hepAGT+/+ mice also had consistent systolic blood pressures [56, 59], suggesting that comparability of data in our blood pressure measurement.

5.2 Highly conserved regions of AGT (Chapter 4)

Our results showed that highly conserved regions, the β-sheet and loop region of AGT synergistically regulate AGT synthesis and consequently AngII-mediated functions, which reveals a novel biological function of these highly conserved regions. Moreover, the loop region might be one of critical region determining the interaction with megalin.
AGT with (1) a single nucleotide mutation on the loop region, (2) triple nucleotide mutations on the loop region, (3) triple nucleotide mutations on the β-sheet, or (4) loop deletion mutation all did not affect blood pressure and atherosclerosis in hepAGT−/− mice fed a Western diet for 12 weeks. The RAS are regulated under different stress conditions, such as high salt intake and low salt intake, which associates with hypertension [187-190]. Thus, whether expression of AGT mutants exerts different effects on AngII-mediated functions in mice fed a high salt diet or low salt diet is another future direction to study the functions of conserved regions of AGT in AGT metabolism and AngII-mediated functions in mice under stress conditions.

Plasma AGT concentration was moderately increased in hepAGT−/− mice infected with AAV encoding AGT with loop deletion, and blood pressure and atherosclerosis in these mice were significantly increased to a comparable level to hpeAGT+/− mice infected with null AAV. However, hepAGT−/− mice infected with AAV encoding AGT with mutation on the β-sheet and loop region failed to repopulate AGT mutant, increase blood pressure and atherosclerosis which were all comparable to hepAGT−/− mice infected with null AAV. The question here is what the exact concentration of AGT can produce AngII which concentration is enough to regulate and affect blood pressure and atherosclerosis. It is difficult to define biological functions which are affected by a specific dose of AGT. To address this question, hepAGT+/− mice could be injected with different doses of GalNAc-conjugated AGT antisense oligonucleotides (ASO) to specifically inhibit AGT expression in hepatocytes. Blood pressure and atherosclerosis could be tested in hepAGT+/− mice expressing with different concentrations of AGT.
In this study, we only tested the interaction of megalin and AGT with a single nucleotide mutation (W292A). There are some difficulties limiting the assessments of the binding affinities of other mutated AGT with megalin. Firstly, megalin is about 600-kD membrane-bound protein [180, 181], which big size causing the difficulty of purification. Secondly, mutations on the β-sheet and loop region at the same time cause the low recombinant AGT protein production.

In the future studies, to solve the problem of purification of megalin, four ligand-binding domains of megalin can be generated separately or generated with different combinations to investigate more detailed interactions between AGT and megalin in vitro. In addition to using purified megalin protein, megalin-expressing cells [191, 192] can be used to study the interaction of AGT with megalin in cells. Retinoic acids and dibutyrl cAMP-treated F9 cells (testicular teratoma stem cell line; ATCC) express megalin (Fig. 5.1). I used recombinant AGT proteins with flag-tag labeling (generated by GenScript) to do AGT uptake experiment in F9 cells to study the interaction of AGT with megalin. However, I failed to get consistent results from cell immunofluorescent staining and western blotting analysis (Fig. 5.2). There were some issues in this experiment: (1) antibody targeting AGT was failed to detect AGT in cells. Also, antibody targeting flag tag did not specifically bind to AGT in western blotting analysis, suggesting that AGT detected in cell immunofluorescent staining by anti-flag antibody might be a non-specific result; (2) how long is enough for AGT uptake into cells is still unclear. Thus, to figure out the availability of specific antibodies targeting recombinant AGT protein and the optimal AGT incubation time and condition is the first thing to do in the future studies.
Figure 5.1 Immunofluorescence confocal microscopy of F9 cells.
F9 cells express megalin after retinoic acids and dibutyrl cAMP incubation for 4 days.
Figure 5.2 Recombinant AGT protein uptake experiments in F9 cells.
F9 cells incubated with retinoic acids and dibutyrl cAMP for 4 days to induce megalin expression, and then incubated with 30 nM recombinant AGT for 30 min or 1 h. (A) Fixed cells were stained with DAPI, anti-megalin antibody, anti-flag antibody or anti-AGT antibody. (B) Cell lysates were used for western blotting analysis. Rabbit anti-flag: IRDye 800CW (green color). Mouse anti-mouse β-actin: IRDye 680RD (red color).
Since megalin is predominantly expressed in renal proximal tubules, mouse renal proximal tubule cell (TKPTS; ATCC) was used to test the expression of megalin. However, megalin was not detected in TKPTS (Fig. 5.3). Although megalin-expressing cells can potentially be used for experiments of AGT and megalin interaction, the further megalin expression confirmation in other cells (e.g. HK-2 cell) and cell culture condition will need to be verified.

**Figure 5.3 TKPTS cells did not express megalin.**
TKPTS cell lysate was used for western blotting analysis. Rabbit anti-human megalin: IRDye 680RD (red color). Mouse anti-mouse b-actin: IRDye 800CW (green color).

In addition, to solve the problem of recombinant AGT protein production, our collaborator, Dr. Vander Kooi in Molecular and Cellular Biochemistry at the University of Kentucky will try to use different cell expression systems to generate recombinant AGT with different mutations for the future studies.
5.3 Perspectives

AGT is the only precursor of all angiotensin peptides, so how AGT is regulated is critical for many pathophysiological functions which mediated by angiotensin peptides. Moreover, targeting AGT acts as a potential therapeutic method of many angiotensin-mediated diseases. For example, maternal liver-derived AGT inhibition by siRNA improved pre-eclampsia phenotypes, such as blood pressure and the fetus growth restriction [70]. In addition, liver-specific AGT inhibition by N-acetylgalactosamine (GalNAc)-conjugated antisense oligonucleotides (ASO) targeting AGT reduced blood pressure in spontaneously hypertensive rats fed an 8 % NaCl diet which is a hypertension model resistant to the RAS inhibitors and protected renal functions in rats salt-deprived which is a model activating renin production and activity [62], showing a promising roles of targeting AGT in treatment of hypertension and kidney injury. Therefore, to understand how AGT function and metabolism are regulated benefits the development of therapy by targeting AGT. Our previous paper demonstrates that AGT and megalin interactions regulate renal RAS homeostasis and development of atherosclerosis [57], indicating the pivotal roles of local renal RAS in AngII-mediated diseases. In our future study, understanding the potential interactions between AGT and megalin would provide evidence how AGT is regulated locally and how local RAS affects AngII-mediated diseases, which might facilitate the development of therapeutic treatment targeting tissue-specific AGT in AngII-associated diseases.
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