Allele-Specific Expression of Angiotensinogen in Human Subcutaneous Adipose Tissue

Sungmi Park, Ko-Ting Lu, Xuebo Liu, Tapan K. Chatterjee, Steven M. Rudich, Neal L. Weintraub, Anne E. Kwitek, Curt D. Sigmund

Abstract—The angiotensinogen gene is genetically linked with hypertension, but the mechanistic basis for association of sequence variants in the promoter and coding region of the gene remains unclear. An E-box at position −20 has been hypothesized to control the level of angiotensinogen expression, but its mechanistic importance for angiotensinogen expression in human tissues is uncertain. We developed an allele-specific polymerase chain reaction–based assay to distinguish among angiotensinogen mRNA derived from variants at the −20 position (rs5050) in the angiotensinogen promoter in adipose tissues obtained during surgery. The assay takes advantage of linkage disequilibrium between the rs5050 (located in the promoter) and rs4762 (located in the coding region) single nucleotide polymorphisms. This strategy allowed us to assess the level of allele-specific expression in A-20C heterozygous subjects comparing the relative proportion of each allele with the total, thus eliminating the problem of variability in the level of total angiotensinogen mRNA among subjects. We show that angiotensinogen mRNA derived from the −20C allele is expressed significantly higher than that derived from the −20A allele in subcutaneous adipose tissue, and increased expression correlates with enriched chromatin binding of upstream stimulatory factor-2 to the −20C E-box compared with −20A. This may be depot selective because we were unable to detect these differences in omental adipose. This provides the first data directly comparing expression of angiotensinogen mRNA and differential transcription factor binding derived from 2 variant alleles in human tissue where the ratio of expression of one allele to another can be accurately determined (Hypertension. 2013;62:41-47.).

Key Words: adipose tissue • angiotensinogen • genetics • hypertension • transcription factors • upstream stimulatory factors

Angiotensinogen (AGT) encodes the precursor for the peptides in the renin–angiotensin system (RAS) and is, therefore, one of the most frequently studied genes for its contributions to hypertension. AGT is synthesized in the liver, adipose, heart, blood vessel, brain, and kidney among others. Its importance in many of these tissues has been well established by animal models where AGT mRNA expression has either been ablated or overexpressed. Adipose tissue AGT has recently become recognized as a contributor to both blood pressure and metabolic regulation. Overexpression of AGT in adipose tissue causes systemic hypertension in mice and favors lipogenesis in cultured human adipocytes, whereas AGT-deficient mice exhibit reduced fat mass and blood pressure. Similarly, adipose-specific AGT-deficiency lowers arterial pressure and blocks the blood pressure elevating effect of a high-fat diet. Therefore, adipose tissue–derived AGT could be a factor contributing to the association between obesity and hypertension.

The primary variant allele of the AGT gene initially investigated in human subjects was M235T. Since then, many associations and case–control studies have failed to provide compelling evidence supporting a pathogenetic role for this variant. There is no difference in the kinetics of the catalytic reaction between renin and either M235 or T235 AGT. Humanized mice carrying the M235 and T235 AGT variants did not exhibit any changes in the level of AGT expression, and only under specific conditions (high salt and dysregulation of renin) was there a difference in arterial pressure. This suggests that M235T may derive its physiological importance from linkage disequilibrium with variants in the AGT promoter. Single nucleotide polymorphisms (SNPs) at nucleotides −6, −20, −217, −517, and −792 in the human AGT promoter have been implicated in the pathogenesis of high blood pressure. We and others reported that the A-20C and G-217A polymorphisms in the human AGT 5′ flanking region strongly influence promoter activity in AGT-expressing cells.

The E-box sequence (CACGTG) overlying a SNP in the rs5050 (located in the promoter) and rs4762 (located in the coding region) single nucleotide polymorphisms. This strategy allowed us to assess the level of allele-specific expression in A-20C heterozygous subjects comparing the relative proportion of each allele with the total, thus eliminating the problem of variability in the level of total angiotensinogen mRNA among subjects. We show that angiotensinogen mRNA derived from the −20C allele is expressed significantly higher than that derived from the −20A allele in subcutaneous adipose tissue, and increased expression correlates with enriched chromatin binding of upstream stimulatory factor-2 to the −20C E-box compared with −20A. This may be depot selective because we were unable to detect these differences in omental adipose. This provides the first data directly comparing expression of angiotensinogen mRNA and differential transcription factor binding derived from 2 variant alleles in human tissue where the ratio of expression of one allele to another can be accurately determined (Hypertension. 2013;62:41-47.).
and USF2. USF1 is a transcription factor that binds to E-box sequences in DNA, as a homodimer or a heterodimer with its partner USF2 to modulate the expression of genes related to glucose and lipid metabolism, including fatty acid synthase (FAS), peroxisome proliferator-activated receptor \( \gamma \) (PPARG or NR1C3), and AGT. Both USF1 and USF2 are essential for AGT transcriptional regulation, and sex-specific mechanisms are involved in their activity on AGT expression in vivo. USF1 polymorphisms were reported to be associated with the level of AGT expression in human fat biopsies. AGT expression in the renal medulla was reported to be higher in subjects carrying the −20C allele than the −20A allele. However, no mechanistic studies have been performed to examine this on an allele-specific basis. We hypothesize that AGT expression derived from the −20C allele is higher in human subcutaneous (SQ) adipose from individuals heterozygous for −20A and −20C alleles. We further tested whether this was attributable to increased binding of USF1 and USF2 to the −20C versus −20A promoter.

Material and Methods

Adipose tissues were collected from SQ and omental depots of patients undergoing surgical procedures at University Hospital, Cincinnati, OH. This study was designed to investigate the mechanism of differential AGT gene expression and transcription factor binding among 2 variant alleles at −20. Thus, we did not collect specific patient-related data for this study. Adipose tissues were collected in cold Dulbecco’s Modified Eagle Medium/F12 medium, transported to the laboratory, and stored at −80°C until used experimentally. All identifying data were removed before shipping the samples to the University of Iowa. The collection protocol was approved by the institutional review board at the University of Cincinnati, and subjects gave informed consent. See the online-only Data Supplement for more detailed procedures for genotyping, measuring gene expression, allele-specific assays, chromatin immunoprecipitation, and statistics.

Results

We obtained 89 human SQ adipose samples and successfully genotyped 76 of them for the rs5050 SNP at position −20 in the AGT promoter. Homozygosity (−20AA) and heterozygosity (−20AC) was identified in 56 (74%) and 20 (26%) samples, respectively. None were homozygous −20CC. This is consistent with the genotype frequencies for white Americans (SQ) adipose from individuals heterozygous for −20A and −20C alleles. We further tested whether this was attributable to increased binding of USF1 and USF2 to the −20C versus −20A promoter.

Analysis of 120 phased chromosomes from the Center d’Étude du Polymorphisme Humain population showed 97% concordance between SNPs rs5050 and rs4762 (Figure 2). SNP rs4762 is a polymorphism at position +620, which was denoted as T174M. We successfully genotyped 58 SQ adipose samples at rs4762. Similar to rs5050, homozygosity (+620CC) and heterozygosity (+620CT) were identified in 41 (71%) and 17 (31%) samples, respectively. Of the 20 samples identified above as −20AC heterozygotes, 17 were also +620CT heterozygotes. Linkage disequilibrium between rs5050 and rs4762 was determined in 16 samples by sequencing cloned fragments, representing the 32 chromosomes encompassing both rs5050 and rs4762. All were confirmed to conform to the predicted −20C/+620T:−20A/+620C haplotypes. The other samples, genotyped as −20C/+620C:−20A/+620C, were deemed uninformative. A summary of the genotype data...
is shown in Table 1. The linkage disequilibrium identified between −20 and +620 was not observed with 2 other SNPs −rs5049 (known as −217) and rs5051 (known as −6) reported previously to influence AGT promoter activity (Table 2).13

We developed allele-specific assays to determine whether AGT mRNA derived from the −20C promoter was expressed higher than AGT mRNA derived from the −20A promoter. We took advantage of the 16 SQ adipose samples that have confirmed linkage disequilibrium between −20C/+620T and −20A/+620C. In other words, expression of AGT mRNA containing +620T could be attributed to the −20C allele, whereas AGT mRNA containing +620C could be attributed to the −20A allele (Figure 2).

Identical primers were used to amplify DNA surrounding rs4762, but different fluorescent probes were used to distinguish +620T from +620C mRNA (Table S1 in the online-only Data Supplement). Varying the amount of cloned +620T or +620C cDNA resulted in a straight line standard curve, and mixing experiments validated the specificity of the assay (Figure S1A; Figure 3A). A similar allele-specific assay centering on rs5050 was developed and validated for the ChIP studies (Figure S1B; Figure 3B). In both assays, there was a slight bias toward the detection of the more frequent +620C and −20A alleles. These represent the hypothesized low expressing alleles.

We measured the relative amount of +620T and +620C mRNA in SQ adipose tissue from 16 subjects heterozygous at the haplotype level using genomic DNA from one of them to develop standard curves (Figure S2). First, we noted a large variability in the total level of AGT mRNA in SQ adipose tissue (Figure 4A). Second, in 15 of 16 samples, the level of AGT mRNA containing +620T (−20C) was higher than AGT carrying +620C (−20A). That the assay is indeed selective is evidenced by the absence of +620T mRNA in 21 samples derived from +620CC homozygotes. On average, 75% of the AGT mRNA in SQ adipose was derived from +620T (−20C), and 25% was derived from +620C (−20A) (Figure 4B).

In addition to SQ adipose, we obtained omental adipose tissue from 8 of the heterozygous +620TC subjects. As for SQ adipose tissue, the total level of AGT expression was higher in omental adipose tissue from −20AC males but not from females, whereas increased expression of both USF1 and USF2 mRNA was identified in samples from −20AC male and female subjects (Figure 5A–5B). Although some individual samples showed enrichment for AGT mRNA carrying +620T (Figure 5C), on the whole this trend was not significant (Figure 5D).

Finally, we tested whether the binding of USF1 and USF2 to the E-box overlying the −20 position in the AGT promoter could explain the increase in AGT mRNA derived from the −20C allele in SQ adipose. ChIP studies revealed that the binding of USF1 was generally quite low (<2-fold enriched

<table>
<thead>
<tr>
<th>Single Nucleotide Polymorphism</th>
<th>rs5050</th>
<th>rs4762</th>
<th>Men</th>
<th>Women</th>
<th>Total</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>CC</td>
<td>11</td>
<td>14</td>
<td>25</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>CC</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>8</td>
<td>9</td>
<td>17</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>CT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Haplotype Summary

<table>
<thead>
<tr>
<th>rs5049 (−217)</th>
<th>rs5050 (−20)</th>
<th>rs5051 (−6)</th>
<th>rs4762 (+620)</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>8</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>4</td>
</tr>
<tr>
<td>G</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td></td>
</tr>
</tbody>
</table>

AGT genotype frequencies in subcutaneous adipose samples are shown. Only shown here are samples that were successfully genotyped at both rs5050 and rs4762.

Haplotypes for the 16 heterozygous samples confirmed to have linkage disequilibrium between rs5050 and rs4762.

Figure 3. Development and validation of allele-specific assays. A mixing experiment using the indicated ratios of rs4762 (620T vs 620C, A) or −rs5050 (20C vs −20A, B) plasmid DNA was used to validate the specificity and selectivity of the assay. Standard curves are shown in Figure S1.
over the immunoglobulin control, Figure 6A), and there was no difference in the relative binding of USF1 to the −20C versus −20A allele (Figure 6C). Binding of USF2 was more robust (Figure 6B) and showed a significant enrichment in binding to −20C (Figure 6C).

**Discussion**

Genetic evidence implicating AGT in hypertension was first reported in 1992.4 The AGT M235T variant was the first examined extensively in association and case–control studies. An extensive literature details studies both supporting or refuting the association with hypertension.7 Our studies using humanized mouse models generally refute the physiological importance of the M235T variant in AGT.9,10 The finding that the 235 and −6 variants were in strong linkage disequilibrium began efforts to identify and examine SNPs in the AGT promoter.19 Nine SNPs in the AGT promoter, some of which overlay transcription factor–binding sites, defined 5 common haplotypes.20 We studied the transcriptional activity of naturally occurring AGT promoter haplotypes containing SNPs at −6, −20, −217, and at 11 other positions in the proximal promoter that in total account for 90% of known diversity. We reported that the −20 and −217 SNPs had the largest influence on AGT transcription and may act cell specifically.13

We focused on the A-20C SNP for several reasons. First, it was reported to be associated with hypertension, cardiac hypertrophy, microangiopathy-related cerebral damage, progression of IgA nephropathy, nonmodulating hypertension, and, most importantly for the present study, may act as a modifier of the relationship between body mass index and blood pressure.21–26 Second, the −20 position lies within an E-box sequence, which binds USF1 and USF2.15 Notably, a naturally occurring splice variant of USF1, which lacks the N-terminal transactivation domain and acts dominant negatively, was reported to repress AGT transcription.27 On the basis of this rationale, we showed that USF1/2 binds more strongly to the −20C than −20A allele in chromatin in AGT-expressing cells, and that ablation of USF1 and USF2 resulted in markedly blunted AGT expression in vivo.18 Similarly, we showed that AGT expression in many tissues was decreased in USF1-deficient mice.16

The main conclusions of the present study are the following: (1) there is great deal of variability in the total level of AGT expression in human adipose tissue, (2) there is an enrichment in the expression of AGT mRNA derived from the −20C allele compared with −20A in SQ adipose tissue from heterozygous subjects, (3) this enrichment seems adipose depot selective because we were unable to detect differences in omental adipose tissue, and (4) there is an enrichment in the binding of USF2 to the −20C allele in chromatin derived from SQ adipose tissue.

Although the total level of AGT mRNA in SQ adipose tissue from −20AA and −20AC females was not different, AGT mRNA derived from the −20C allele was higher than that derived from the −20A allele in heterozygous subjects. It is difficult to explain this apparent discrepancy, but it suggests that other factors may play an important role in AGT regulation, and that the total level of AGT is a result of the sum of all these factors. Consistent with this, the sequence surrounding −20A has been reported to form an estrogen response element that causes increased transcriptional activity in cells cotransfected with estrogen receptor-α (ERα).11 It is unclear whether there are mechanisms that would induce a greater ERα–dependent effect in −20AA versus −20AC women. Along these lines, plasma levels of AGT are higher in pregnant women homozygous for the −20A allele, intermediate for heterozygous women, and the lowest in women homozygous for the −20C allele.24 That the effect of ERα on AGT expression of alleles carrying −20A may be antagonized by the binding of the orphan transcription factor ARP-1 further complicates the mechanisms of AGT regulation by variants at this site.29 Of course, because the samples were deidentified, we are unable to determine whether they came from pre- or postmenopausal women.

It is also interesting that the allele-specific increase in expression of −20C AGT mRNA observed in SQ adipose tissue was not replicated in omental adipose, despite an increase in total AGT mRNA in omental adipose in −20AC versus −20AA
male subjects. The reason for this discrepancy is unclear. The simplest explanation may be that sample size of omental adipose tissue available for our study was too small to detect differences. Although the power to detect differences in the SQ data set was 1.0 (with $\alpha=0.5$), the power to detect differences in the omental data set was only 0.162. This is not surprising, given the overall variability in $AGT$ expression observed among subjects. Alternatively, the data could suggest that there is some cell-specificity in mediating the transcriptional responses to variants in the $AGT$ promoter. Indeed, we previously reported differences in the transcriptional activity of common haplotypes of the $AGT$ promoter in many cell types of diverse origin. It remains unclear whether these differences could extend to different types of adipose cells or adipose cells derived from different depots. Along these lines, it is interesting to note that both endogenous mouse $AGT$ mRNA and human $AGT$ mRNA (derived from a transgene) increased in omental, reproductive, and perirenal adipose, but not SQ or brown adipose, in response to high-fat diet in mice. Thus, there seems to be some selectivity in mediating $AGT$ transcriptional responses to physiological cues among adipose depots. It is also possible that this reflects a second tier of regulation. With this regard, Chatterjee et al identified that histone deacetylase 9 (HDAC9) can bind to the USF1/USF2 complex at the C/EBP$\alpha$ (CAAT enhancer-binding protein alpha) promoter to repress transcription of the C/EBP$\alpha$ gene, a major regulator of adipogenic differentiation. HDAC9 expression is higher in omental than SQ adipose tissue, and this correlates with

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Quantification of +620T and +620C containing mRNA in omental adipose. **A** and **B,** Expression of angiotensinogen ($AGT$), upstream stimulatory factor ($USF1$), and $USF2$, in omental adipose tissues from men (**A**) and women (**B**). *P*<0.05 vs −20AA. All data presented as mean±SEM. **C,** The amount of mRNA carrying +620T and +620C is plotted. The line of identity (representing equal amounts of both alleles) is shown by the long dashed line. The regression line (calculated to the origin) derived from the combined male and female +620CT data is shown in the short dashed line. **D,** Individual samples in **C** were transformed to a percentage of total $AGT$ mRNA and plotted. There is no significant difference in the relative amount of +620C vs +620T as calculated by a 1-sample $t$ test using 50% as the hypothetical mean.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Binding of upstream stimulatory factor ($USF1$) and $USF2$ to −20 chromatin. **A** and **B,** ChIP analysis was used to measure the enrichment of $USF1$ and $USF2$ vs immunoglobulin ($IgG$) in the chromatin precipitate with antisera targeting each transcription factor. The relative binding was determined by quantitative polymerase chain reaction using allele-specific probes and standard curves similar to those shown in Figure 3B. The background of the assay, that is, where the ChIP signal equals the $IgG$ control, is indicated by the dashed line. **C,** Quantification of the relative percentage of $USF1$ and $USF2$ binding to −20A vs −20C chromatin. Significance was determined by paired $t$ test comparing −20A vs −20C. All data are presented as mean±SEM.
decreased capacity to undergo adipogenic differentiation. On induction of adipogenic differentiation, the level of HDAC9 decreases and is replaced on the USF1/2 complex with the transcriptional coactivator p300 histone acetyl transferase. Thus, it will be interesting in future studies to determine whether USF1/2 bound to the −20 region of the AGT promoter is differentially complexed with HDAC9 or p300 histone acetyl transferase in omental and SQ adipose tissue.

One of the strengths of the present study was linking the allele-specific AGT expression data with the level of USF1 and USF2 binding. On the basis of our previous results of increased USF1, but not USF2 binding to plasmids carrying −20C transected in HepG2 cells, we were surprised that it was USF2 that showed modestly enriched binding to the −20C allele in chromatin from SQ adipose tissue. This may be attributable to the observation that the binding of USF1 was quite low, essentially at the detection threshold of the assay defined by the immunoglobulin control. Indeed, the ChIP assays on human adipose tissue were much more difficult and less sensitive than our previous experience with cultured cells. Ultimately, given these results, it may be interesting to optimize the assay or develop immortalized cells, so that a greater number of mechanistic studies can be performed. It would be also interesting to compare the binding of Erα and ARP-1 with USF1/2.

Perspectives and Significance

Whether the AGT gene plays an important role in the genetics of hypertension has been and will be debated for years. What is beyond dispute is the importance of the RAS in the regulation of blood pressure and the use of RAS blockers as therapy. The -20 region of the AGT promoter differentially complexes with HDAC9 or p300 histone acetylation in omental and SQ adipose tissue.

The -20 region of the AGT promoter is differentially complexed with HDAC9 or p300 histone acetyl ation in omental and SQ adipose tissue. This may be attributable to the observation that the binding of USF1 was quite low, essentially at the detection threshold of the assay defined by the immunoglobulin control. Indeed, the ChIP assays on human adipose tissue were much more difficult and less sensitive than our previous experience with cultured cells. Ultimately, given these results, it may be interesting to optimize the assay or develop immortalized cells, so that a greater number of mechanistic studies can be performed. It would be also interesting to compare the binding of Erα and ARP-1 with USF1/2.

Acknowledgments

We acknowledge the DNA Core Facility at the University of Iowa for assistance with DNA sequencing and genotyping.

Sources of Funding

This work was supported by National Institutes of Health grants HL048058, HL061446, HL062984, HL084207 to Dr Sigmund; HL076684 and HL112640 to N.L. Weintraub; and HL089895 and DK089417 to A.E. Kwitek. The authors gratefully acknowledge the generous research support of the Roy J. Carver Trust (to Dr Sigmund) and the Fraternal Order of Eagles Diabetes Research Center (to A.E. Kwitek).

Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

- We took advantage of linkage disequilibrium to develop allele-specific assays designed to measure the amount of angiotensinogen (AGT) mRNA derived from single nucleotide polymorphisms in the AGT promoter.
- Measurement of AGT mRNA derived from AGT promoter single nucleo-tide polymorphisms in adipose tissue of subjects heterozygous for the single nucleotide polymorphism.
- In subcutaneous but not omental adipose tissue, expression of AGT mRNA derived from the −20C allele of the AGT promoter is higher than that from the −20A allele in heterozygous subjects carrying both alleles.
- Increased expression correlated with increased binding of upstream stimulatory factor-2 to the −20C E-box.

**What Is Relevant?**

- The AGT gene has been genetically linked with hypertension.
- Genetic variants in both the promoter and coding region of the AGT gene are hypothesized to be significant, but the mechanistic basis for this remains unclear.
- An E-box at position −20 binds upstream stimulatory factor-1 and upstream stimulatory factor-2 and has been hypothesized to control the level of AGT expression, but mechanistic studies of AGT expression in human tissues are lacking.

**Summary**

This provides the first data directly comparing expression of AGT mRNA and differential transcription factor binding derived from 2 ariant alleles in tissues from human subjects. Our study is sig-nificant in that we demonstrate that the level of AGT expression in human SQ adipose tissue may, at least in part, be genetically determined.
Allele-Specific Expression of Angiotensinogen in Human Subcutaneous Adipose Tissue
Sungmi Park, Ko-Ting Lu, Xuebo Liu, Tapan K. Chatterjee, Steven M. Rudich, Neal L. Weintraub, Anne E. Kwitek and Curt D. Sigmund

Hypertension. 2013;62:41-47; originally published online May 6, 2013;
doi: 10.1161/HYPERTENSIONAHA.113.01330

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/62/1/41

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2013/05/06/HYPERTENSIONAHA.113.01330.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENTAL MATERIAL

Allele-Specific Expression of Angiotensinogen in Human Subcutaneous Adipose Tissue

Sungmi Park¹, Ko-Ting Lu¹, Xuebo Liu¹, Tapan K. Chatterjee², Steven M. Rudich³, Neal L. Weintraub², Anne E. Kwitek¹ and Curt D. Sigmund¹

¹Department of Pharmacology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa
²Department of Internal Medicine, University of Cincinnati, Cincinnati, Ohio
³Department of Surgery, Wright State University, Dayton, Ohio
DETAILED METHODS

**Genotyping.** We isolated genomic DNA from 20 mg of frozen adipose tissue using phenol/chloroform extraction. Two separate PCR reactions were used to amplify genomic DNA from -314 to +76 and from +472 to +794 using the primers described in Table S1 to genotype SNPs rs5050 (A-20C) and rs4762 (C620T). Sequencing was performed by the University of Iowa DNA Core. Chromosome-specific haplotypes were amplified (~4.4kb) and cloned from genomic DNA from heterozygous individuals using TA cloning. The DNAs were then sequenced to assess the level of linkage disequilibrium between rs5050 and rs4762 SNPs (-20C/620T vs -20A/620C). In some cases, where the sequence data was not of sufficient quality, the genotype was determined by using the SNP specific PCR assay on AGT cDNA as described below. The sex of the sample was determined by PCR amplification of the SRY gene in genomic DNA isolated from the adipose samples.

**Gene expression.** Total RNA was extracted from frozen human adipose samples using TRIzol Reagent according to the protocol provided by the manufacturer (Invitrogen) and QIAGEN RNeasy Mini kit’s manual. The cDNA generated by Superscript III (Invitrogen) was used for real-time PCR. Expression levels were assessed by quantitative real-time RT-PCR using TaqMan primer/probe sets from Applied Biosystems (AGT, Hs01586213_m1; USF1, Hs00273038_m1; USF2, Hs00231528_m1; FASN, Hs01005622_m1; PPARG, Hs01115513_m1). Data were normalized to either β-actin (ACTB) or HPRT1 (Applied Biosystems) and quantified by the $2^{-\Delta\Delta CT}$ method.¹

**Allele-specific analysis.** We designed allele-specific probes for rs5050 (-20) and rs4762 (+620) using the search engine at Biosearch Technologies (Novato, CA). Custom made fluorescent probes were ordered from Biosearch Technologies, whereas PCR primers were ordered from IDT (Coralville, IA). Primer and probe sequences are in Table S1. A standard curve was generated with known amounts of cloned genomic DNA representing each haplotype. For quantification of allele specific mRNA expression in heterozygotes, reverse transcription (RT) using 1µg of RNA in 20µl volume was carried out with the SuperScript first-strand synthesis system as described by the manufacturer (Invitrogen). One µl of cDNA from SQ RT product was utilized with A Fast Taqman Gene Expression Master (Life Technologies) mix in a StepOnePlus (Applied Biosystems, CA). The amount of each haplotype specific mRNA was determined by quantitative real-time PCR compared with the standard curve using the custom primers and probes for rs4762.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChIP) was performed in frozen adipose samples using the protocol originally designed for human pluripotent stem cells as described previously. Antibodies against USF1 (sc-229X, Santa Cruz Biotechnology), USF2 (sc-861X, Santa Cruz Biotechnology) or rabbit IgG (sc-2027, Santa Cruz Biotechnology) were incubated with sonicated chromatin and Dynabeads magnetic beads (Life Technologies, CA) overnight at 4°C. Immunoprecipitated genomic DNA was amplified using A Fast Taqman Gene Expression Master Mix in
StepOnePlusTM (Applied Biosystems, CA) using the primers and probes designed for rs5050 (Table S1).

Statistics. Data satisfying both normality and equal variance tests were analyzed by parametric t-tests/ANOVAs as appropriate, and were expressed as mean ± SEM. Data failing either normality or equal variance tests were analyzed by equivalent non-parametric tests. Statistical significance was defined at a value of P<0.05.
Supplemental Reference List


## Supplemental Table

### Table S1: Summary of Primers and Probes

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Promoter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-314 → +76</td>
<td>CTCAGGCTGTCA</td>
<td>AGAGACAAGACCG</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>CACACCTA</td>
<td>AGAAGGAG</td>
<td></td>
</tr>
<tr>
<td>Coding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+472 → +794</td>
<td>CTACAGGCAATC</td>
<td>CAGCCAGTCTTCC</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>CTGGGTGT</td>
<td>ATCCTGT</td>
<td></td>
</tr>
<tr>
<td>rs4762 (T allele)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+578 → +759</td>
<td>CCCAGGGCAGG</td>
<td>TCAGCAGCAACAT</td>
<td>5’ d CALFluorOrange560-pdCdUgpCdUgpCdUpdCpdCApdUGgpUG-BHQ-1 plus 3’</td>
</tr>
<tr>
<td></td>
<td>GCTGATAG</td>
<td>CCAGTTC</td>
<td></td>
</tr>
<tr>
<td>rs4762 (C allele)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+578 → +759</td>
<td>CCCAGGGCAGG</td>
<td>TCAGCAGCAACAT</td>
<td>5’ d FAM-pdUGpCdUgpUpdCpCdCpApdCGgpU-BHQ-1 plus 3’</td>
</tr>
<tr>
<td></td>
<td>GCTGATAG</td>
<td>CCAGTTC</td>
<td></td>
</tr>
<tr>
<td>rs5050 (C allele)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Promoter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-66 → +44</td>
<td>CAGCCTGGGAA</td>
<td>CCGGCTTACCTTC</td>
<td>5’d FAM-AGpCdUApdUAApdUAGGgpCdUpdCGpU-BHQ-1 plus 3’</td>
</tr>
<tr>
<td></td>
<td>CAGCTCCAT</td>
<td>TGCTGTAG</td>
<td></td>
</tr>
<tr>
<td>rs5050 (A allele)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Promoter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-66 → +44</td>
<td>CAGCCTGGGAA</td>
<td>CCGGCTTACCTTC</td>
<td>5’d FAM-AGpCdUApdUAAApdUAGGpCApUpdCG-BHQ-1 plus 3’</td>
</tr>
<tr>
<td></td>
<td>CAGCTCCAT</td>
<td>TGCTGTAG</td>
<td></td>
</tr>
</tbody>
</table>
Figure S1. Standard curves for rs4762 (A) and rs5050 (B) SNPs were developed with known amount of plasmid DNA carrying each SNP.
Figure S2. Standard curves were generated using a known amount of genomic DNA from a +620CT heterozygote. Detection was with custom made allele-specific probes labeled with either CAL Fluor Orange (rs4762T, A) or FAM (rs4762C, B). Primer and probe sequences are indicated in Table S1.