Angiotensinogen Gene Polymorphism at -217 Affects Basal Promoter Activity and is Associated with Hypertension in African-Americans

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Summary

Hypertension is a serious health problem in Western society, in particular for the African-American population. Although previous studies have suggested that the angiotensinogen (AGT) gene locus is involved in human essential hypertension, the molecular mechanisms involved in hypertension in African-American population remain unknown. We show that an A/G polymorphism at -217 in the promoter of the AGT gene plays a significant role in hypertension in African-American population. The frequency of -217A allele is increased significantly in African-American hypertensive subjects as compared to normotensive controls. We also show that the nucleotide sequence of this region of AGT gene promoter binds strongly to the C/EBP family of transcription factors when nucleoside A is present at -217. In addition, we show that reporter constructs containing human AGT gene promoter with nucleoside A at -217 have increased basal transcriptional activity on transient transfection in HepG2 cells as compared to reporter constructs with nucleoside G at -217. Finally, we show that IL-6 treatment in the presence or absence of over-expressed C/EBPβ increases the promoter activity of reporter constructs containing nucleoside A at -217 as compared to reporter constructs containing nucleoside G at -217. Since, the AGT gene is expressed primarily in liver and adipose tissue and C/EBP family of transcription factors plays an important role in gene expression in these tissues, we propose that increased transcriptional activity of the -217A allele of the human AGT gene is associated with hypertension in African-Americans.
Key Words: Hypertension, African-Americans, angiotensinogen gene, single nucleotide polymorphism, allele frequency, C/EBP, transient transfection, gel shift assay, HepG2 cells.
Hypertension is a serious risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure (1-3). It is estimated that hypertension affects 50 million Americans with a prevalence rate of 25-30% in the adult Caucasian population and the incidence of hypertension is even greater in the African-American population. Hypertension is a polygenic disease and it has been estimated by segregation analysis and twin studies that approximately 45% of the inter-individual differences in blood pressure can be accounted by genetic differences. However, molecular mechanisms involved in the pathophysiology of human hypertension remain unknown. The renin-angiotensin system plays an important role in the regulation of blood pressure and the octapeptide, angiotensin-II, is one of the most active vasopressor agents (4,5). Angiotensin-II is obtained from its precursor molecule, angiotensinogen (AGT), which is primarily synthesized in the liver and adipose tissue, and to a lesser extent in the kidney, brain, heart, adrenal, and vascular walls (6,7). AGT is first converted by renin to produce a decapeptide, angiotensin-I, which is then converted to angiotensin-II by the removal of a C-terminal dipeptide by angiotensin-converting enzyme (ACE). In experimental, as well as clinical studies, administration of renin-angiotensin inhibitors is effective in reducing blood pressure and end organ damage (8).

Jeunemaitre et al., (9) have used a highly polymorphic CA dinucleotide marker located in the 3'-region of the human AGT gene and shown an association of this gene with essential hypertension in Caucasian population by linkage analysis. This association was later confirmed in Japanese hypertensive subjects (10). On the other hand, no association or linkage was found between genes of other components of the renin-angiotensin system
namely renin (11), angiotensin converting enzyme (12) or angiotensin-II subtype 1 receptor (13) with human hypertension. Jeunemaitre et al., (9) have also shown that the molecular variant M235T of AGT gene is associated with increased plasma AGT level. However, since amino acid 235 is located far away from the renin cleavage site, this polymorphism does not explain the mechanism involved in increased plasma AGT level. The human AGT gene also has an A/G polymorphism at -6. It has been shown recently that: (a) molecular variants 235T and -6A are in complete linkage disequilibrium and (b) reporter constructs containing human AGT gene promoter with nucleoside A at -6 have increased promoter activity on transient transfection in human liver derived HepG2 cells compared to reporter constructs containing nucleoside G at -6 (14). Results of these experiments suggest that increased plasma AGT level by allele 235T may be due to increased transcriptional activity of the human AGT gene by nucleoside A at -6.

Although hypertension is more prevalent in the black population and complication rates, particularly for renal failure, are many times higher in blacks than whites, relatively little work has been done to understand the molecular mechanism involved in hypertension in this population. Plasma AGT level is generally higher in black population (15). It has been shown that: (a) plasma AGT level is about 19% higher in black children as compared to white children, (b) blood pressure is normally higher and increases faster over time in black children as compared to white children, and (c) plasma AGT level is associated with AGT gene in black children (16-18). Caulfield et al., have found an association between the AGT gene locus and high blood pressure in 63 affected sibling pairs of African-Carribbean origin using CA dinucleotide marker (19). However, these
workers could not find an association between variants M235T or A/G at -6 and hypertension in African-American population. Other studies have also suggested that although the frequency of -6A allele is increased in African-American population, there is no association between -6A allele and hypertension in this population (20).

Our laboratory is interested in understanding the role of single nucleotide polymorphisms (SNPs) in the AGT gene on human hypertension. The nucleotide sequence of the human AGT gene promoter contains an A/G polymorphic site at -217. In this paper we show that the -217A allele of the AGT gene is associated with hypertension in African-American population (p=.0017) and not in Caucasian population (p=.12). The nucleotide sequence of the human AGT gene containing the -217 A/G polymorphic site has partial homology with a consensus C/EBP binding site. We show that an oligonucleotide containing human AGT gene promoter with nucleoside A at -217 binds more strongly to recombinant C/EBPα, C/EBPβ and DBP. In addition, we show that reporter constructs containing human AGT gene promoter with nucleoside A at -217 have increased basal promoter activity on transient transfection in HepG2 cells as compared to reporter constructs containing nucleoside G at -217. Furthermore, we show that IL-6 treatment in the presence or absence of over-expressed C/EBPβ increases the promoter activity of reporter constructs containing nucleoside A at -217 as compared to reporter constructs containing nucleoside G at -217.
EXPERIMENTAL PROCEDURES

Plasmid construction-Reporter construct pHAGT1.3\textit{luc} was constructed by PCR amplification of human AGT gene\cite{21,22} using TATGCTAGTCGAGTGAGTCCCTATCTATAGTGAACA as the forward primer and CAAGTACCAGTAAGTGAGTCTGA GTGGGGCCCCCGCTTA as the reverse primer. The amplified fragment contained the nucleotide sequence -1206 to +70 and was subcloned in the pGL3 basic vector that lacks eukaryotic promoter and enhancer sequences (Promega, Madison, WI). Reporter construct pHAGT303\textit{luc} was constructed by PCR amplification of human AGT gene\cite{22} using ACACACCTAGGGAGATGCTCCCGTTTCTGG as the forward primer and CAAGTACCAGTAAGTGAGTCTGA GTGGGGCCCCCGCTTA as the reverse primer. The amplified fragment contained the nucleotide sequence -303 to +70 and was subcloned in the pGL3 basic vector. These reporter constructs had nucleoside A at -6 and -217. Nucleoside A at -217 in these reporter constructs was mutated to G by site specific mutagenesis using CCTGCACCAGTCTCACTCTGTTCAGTCAGTG and its complementary oligonucleotide by Stratagene kit (Stratagene, La Jolla, CA). Nucleotide sequences of mutated reporter constructs were confirmed by sequence analysis. Reporter constructs (223A)$_2$\textit{luc} and (223G)$_2$\textit{luc} were constructed by dimerization of oligonucleotides CCTGCACCAGTCTCACTCTGTTCAGTCAGTG and CCTGCACCGGCTCACTC TGTTCAGTCAGTG (position of A/G polymorphic site is underlined) and blunt ended ligation of dimers in the SmaI site of pGL3 promoter vector. PGL3 promoter vector contains SV40 promoter but not the enhancer sequence upstream
of the luciferase gene. Expression vector RSV-β-gal was obtained from Promega. Restriction enzymes were purchased from New England Biolab (Beverly, MA). Plasmid DNAs for transient transfection were prepared by Qiagen midi or maxi plasmid kits (Chatsworth, CA) using conditions described by the manufacturer. PolyFect transfection reagent was also purchased from Qiagen.

**Cell Culture and transient transfection-** Human hepatoma cells (HepG2) were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in an atmosphere of 5% CO₂. For transient transfections, reporter DNA (1.0 µg), and RSV β-gal DNA (0.05 µg) were mixed with pBluescript DNA to a final weight of 1.5 µg of DNA. Transient transfections were performed using the manufacturer protocol. For co-transfection experiments, expression vectors containing MSV-C/EBPβ and -δ (0.2 µg) were added to the reporter constructs. After 24 h of transfection, cells were treated for an additional 24 h with recombinant human IL-6 (10 ng/ml of the media). Cells were harvested 48 h post-transfection and whole cell extracts were prepared by resuspension in 100 µl of lysis buffer (Promega). An aliquot of the cell extract was used to measure luciferase activity by Turners Design Luminometer TD 20/20 using a luciferase assay system (Promega) as described by the manufacturer. Luciferase activity was normalized with the β-gal activity. β-gal activity was determined as described previously (23).

**Gel Mobility Shift Assay -** The probes for electrophoretic mobility shift assay (EMSA) were chemically synthesized, annealed and radiolabeled at the 5'-ends by polynucleotide
kinase using \([\gamma^{32}P]\) ATP. DNA fragments (20,000-50,000 cpm), 1-2 µg of poly(dI-dC), and 5-10 µg of the nuclear extract or 10-20 ng of recombinant proteins were incubated in a solution containing 10 mM HEPES (pH 7.5) - 50 mM KCl - 5 mM MgCl₂ - 0.5 mM EDTA - 1 mM DTT - 12.5% glycerol in ice for 30 min and separated on a 5-8% polyacrylamide gel in a cold room. After 2-3 h, the gel was dried under vacuum and protein-nucleic acid complexes were identified by autoradiography. For supershift assay, 1 µl of antibody was added to the reaction mixture that was incubated for 30 min and analyzed by EMSA. Radioactive oligonucleotides were purified by polyacrylamide gel electrophoresis followed by electroelution for quantitative gel shift assay. Nuclear extracts for gel mobility shift assays were prepared by modification of a previously described method (24). Recombinant C/EBPα and C/EBPβ were obtained through bacterial expression of histidine-tagged proteins as described previously (25). Recombinant DBP was obtained using an \textit{in vitro} coupled transcription-translation system obtained from rabbit reticulocytes as described previously (23). Antibodies against C/EBPα and C/EBPβ were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

\textbf{Oligonucleotides}- Double stranded oligonucleotides 223A and 223G were obtained by annealing \textit{CGACCCTGCACCGCTCACTCT} and \textit{CGACCCTGCACCAGCTCACTCT} with their respective complementary oligonucleotides. Double stranded oligonucleotides containing consensus C/EBP, NF-1, and HNF-3 binding sites were obtained by annealing \textit{AGTATTGTGCAATGT}, \textit{CCTTTGGCATGCTGCCAATTG}, and \textit{TATTATTGACTTAGTGATC} with their respective complementary
oligonucleotides.

**Patient Selection**- We studied 186 African-American and 127 Caucasian subjects with hypertension (mean age: 59 ± 10 years) and 156 African-American and 135 Caucasian normotensive controls (mean age 58 ± 10 years). All of these subjects were recruited from the outpatient department of The State University of New York Health Science Center at Brooklyn, NY and Westchester Medical Center, Valhalla, NY. All case and control subjects gave informed consent before participating in the research. The research protocol was approved by the Institutional Review Board at New York Medical College, Valhalla, NY. All cases were diagnosed as having essential hypertension. The criteria for hypertension was defined as a systolic blood pressure (SBP) > 140 mmHg, a diastolic blood pressure (DBP) > 90 mmHg, or under antihypertensive therapy. Blood pressure was measured twice with the subject seated with a 5 minute of interval between measurements. The normotensives (with SBP/DBP < 140/90 mmHg) without a history of hypertension were recruited from the same population and matched for the sex and age. All participants completed a standard questionnaire on personal medical history and family history of hypertension.

**Analysis of the Genomic DNA**- The genomic DNA from hypertensive and control subjects was amplified using CTCAGTGCTGTCACACACCTA as the forward primer and AAGTGACACCACCTCCAGTCTTTAGT as the reverse primer. The amplification product (233 bp) contained the nucleotide sequence -314 to -82 of the human AGT gene promoter including A/G polymorphic site at -217. These amplified fragments were
treated either with Alu I or Hpa II to identify A/G polymorphic site at -217. Restriction enzyme Alu I (restriction site AGCT) cuts the amplified sequence if nucleoside A is present at -217 and produces 134 and 99 bp fragments. On the other hand restriction enzyme HpaII (restriction site CCGG) cuts the amplified fragment if nucleoside G is present at -217 and produces 136 and 97 bp fragments. After restriction analysis, resulting fragments were separated by 3.5% agarose gel electrophoresis. The nucleotide sequence of amplified products was determined by sequence analysis to confirm the results of restriction analysis.

Statistical Analysis- The Graphpad statistical software package (Graphpad 3.00 for Windows, GraphpadSoftware, San Diego, CA) was used for analysis of the clinical characteristics, differences in allele frequency between case and control subjects and comparison of promoter activity of different reporter constructs in transient transfection assays. Baseline characteristics between hypertensive and normotensive subjects were compared using unpaired t tests or Fisher’s exact test for contingency table where appropriate. Genetic data were analyzed for allele frequency by gene-counting method. Hardy-Weinberg equilibrium was tested by using the computer program GDA (http://lewis.eeb.uconn.edu/lewishome/software.html). (Lewis, P.O, and Zaykin, D., 2001. Genetic Data Analysis: Computer program for the Analysis of Allelic Data) (Version 1.0 d16c). Genotype distribution and differences in allele frequencies between case and control subjects were compared using Fisher’s exact test for contingency table since all the variants are dichotomous. Odds ratio with 95% confidence intervals estimated the relative risk for hypertension associated with –217A allele carrier. Unpaired
t tests were performed to compare relative luciferase activities of reporter constructs containing nucleoside A or G at position -217 of AGT gene promoter in transfection experiments. All experiments were conducted in sextuplicate in four independent transfections as described recently (26).
Results

Frequency of -217A allele of AGT gene is increased in African-American hypertensive patients

To understand the role of A/G polymorphism at -217 in the promoter of AGT gene in hypertension, we have analyzed genomic DNA from 186 hypertensive and 156 normotensive African-American subjects. All patients and control subjects were in Hardy-Weinberg equilibrium. The genomic DNA was amplified by PCR and the product was analyzed for the A/G polymorphic site at -217 by restriction analysis (Fig. 1). The frequency of the -217A allele in hypertensive patients was 0.29 as compared to 0.19 in normotensive population which is highly significant (p= 0.0017 and OR =1.792) (Table I). To compare the role of this polymorphic site on hypertension in the African-American and Caucasian populations, we also analyzed genomic DNA from 127 Caucasian hypertensive subjects and 135 normotensive controls. The frequency of -217A allele in Caucasian hypertensive subjects was 0.15 and in normotensive controls was 0.11 which is not significant (p= .12) (Table I). Statistical analysis based on -217 A/G genotype (using A allele as a dominant model) also suggested a significant role of the -217A allele in hypertension in African-Americans (p= 0.0021 and OR = 2.015) and not in Caucasians (Table II). Since an A/G polymorphism at -6 has been previously associated with hypertension, we also analyzed genomic DNA from the African-American and Caucasian populations for this polymorphism. The frequency of -6A allele was 0.87 in African-American hypertensive subjects and 0.85 in normotensive controls which was not significant (p=0.58) (Table III). However, the frequency of -6A allele was marginally significant in Caucasian subjects (p=0.06). These experiments suggested that -217A allele
of the human AGT gene plays a significant role in essential hypertension in African-Americans and not in Caucasians.

**Reporter constructs containing human AGT gene promoter with nucleoside A at -217 have increased basal promoter activity on transient transfection in HepG2 cells as compared to reporter constructs containing nucleoside G at -217**

In order to understand the role of A/G polymorphism at -217 on transcriptional regulation of the human AGT gene, we performed transient transfection of reporter constructs pHAGT1.3luc and pHAGT303luc containing either nucleoside A or G at -217 in HepG2 cells. The promoter activity was analyzed after 48 h of transfection and normalized with the β-gal activity. Results of this experiment (Fig. 2) suggested that reporter construct pHAGT1.3luc with nucleoside A at -217 gave a 24% increase in the basal promoter activity as compared to the reporter construct pHAGT1.3luc with nucleoside G at -217 (p<.001). On the other hand reporter construct pHAGT303luc with nucleoside A at -217 gave a 37% increase in the basal promoter activity as compared to the reporter construct pHAGT303luc with nucleoside G at -217 (p<.001). We also synthesized reporter constructs where two copies of an oligonucleotide containing nucleotide sequence -225 to -196 of the human AGT gene promoter with either nucleoside A or G at -217 were ligated in front of the luc gene in pGL3 promoter vector. These reporter constructs were then used in transient transfection assay in HepG2 cells. Results of this experiment indicated that the reporter construct with nucleoside A at -217 had 84% increased basal promoter activity as compared to the reporter construct containing nucleoside G at this
position. Taken together, these experiments suggest that nucleoside A at -217 increases the basal promoter activity of reporter constructs containing the human AGT gene promoter on transient transfection in HepG2 cells as compared to nucleoside G at -217.

Recombinant C/EBP family of transcription factors bind strongly to an oligonucleotide containing nucleoside A at -217 as compared to the same oligonucleotide containing nucleoside G at -217

The nucleotide sequence of human AGT gene promoter (located between -217 and -225) has partial homology with the C/EBP binding site (Fig. 3A). The consensus C/EBP binding site TT/GNNNGCAAT/G (shown in the reverse orientation in line 2) has one mismatch when nucleoside A is present at -217 and two mismatches when nucleoside G is present at -217. In order to examine whether this region of the human AGT gene binds to the C/EBP family of transcription factors, we performed gel shift assays using oligonucleotides 223A and 223G in the presence of recombinant C/EBPα that was synthesized as a His-tagged protein. Results of this experiment are presented in Fig. 3B. Lane 1 shows the gel shift assay in the presence of r-C/EBPα in the absence of competitor DNA, lane 2 shows the same assay in the presence of a 100 fold excess of cold oligonucleotide 223A, and lane 3 shows the assay in the presence of a non-specific cold oligonucleotide containing consensus NF-1 binding site. Lane 4 shows the assay in the presence of C/EBPα antibody and lane 5 shows the assay in the presence of pre immune serum. Lanes 6-10 show the same reactions in the presence of oligonucleotide 223G. Results of this experiment indicate that oligonucleotide 223A (containing
nucleoside A at -217) forms a specific complex with rC/EBPα and the intensity of this complex is at least 10 fold greater when compared to the complex formed with oligonucleotide 223G (containing nucleoside G at -217).

We next performed a gel shift assay using oligonucleotides 223A, 223G and an oligonucleotide with consensus C/EBP binding site in the presence of recombinant C/EBPβ (which was also synthesized as a His-tagged protein). Results of this experiment (Fig. 4) show that the oligonucleotide 223A forms a complex with r-C/EBPβ (lane 1) which was supershifted in the presence of C/EBPβ antibody (lane 2). An oligonucleotide containing consensus C/EBP binding site also formed a similar complex with r-C/EBPβ (lane 3) that was partially supershifted in the presence of C/EBPβ antibody (lane 4). In order to compare the binding of r-C/EBPβ to 223A and 223G, we performed a gel shift assay in the presence of equal amount of purified radioactive oligonucleotides using two concentrations of r-C/EBPβ. Results of this experiment show that r-C/EBPβ formed a much stronger complex with 223A as compared to 223G (compare lanes 5 and 6 with lanes 7 and 8). As a control, we also performed a gel shift assay with 223A and 223G using two concentrations of r-C/EBPα (compare lanes 9 and 10 with lanes 11 and 12). Results of this experiment confirmed our previous observation that oligonucleotide 223A forms a stronger complex with r-C/EBPα as compared to oligonucleotide 223G.

Since DBP also plays an important role in transcriptional regulation of liver specific genes especially during circadian rhythm and binds to C/EBP binding sites (27), it was of interest to determine whether DBP also binds to this region of the human AGT gene
promoter. To answer this question, recombinant DBP was synthesized by in vitro coupled transcription-translation using a rabbit reticulocyte system. Recombinant DBP was then used in a gel shift assay with equal amount of purified radioactive oligonucleotides 223A and 223G. Results of this experiment are shown in Fig 5. Lane 1 shows the reaction in the presence of oligonucleotide 223A and lane 2 shows the reaction in the presence of oligonucleotide 223G. Lane 3 shows the reaction of 223A and r-DBP in the presence of DBP antibody, lane 4 shows the same reaction in the presence of a non-specific NF-1 antibody. Lane 5 shows the reaction in the presence of a 100 fold excess of an oligonucleotide containing consensus C/EBP binding site, and lanes 6 and 7 show the reaction in the presence of 100 fold excess of oligonucleotides containing HNF3 and NF-1 binding sites respectively. Results of this experiment indicated that the DBP antibody produced a supershift (faint band shown by a broken arrow) whereas NF-1 antibody had no effect. In addition, cold C/EBP oligonucleotide reduced the intensity of this complex but cold oligonucleotides containing NF-1 and HNF3 consensus binding sites did not compete with the complex. Taken together, results of this experiment suggested that oligonucleotide 223A formed a much stronger complex with r-DBP as compared to oligonucleotide 223G.

**IL-6 treatment in the presence or absence of C/EBPβ increases the promoter activity of reporter constructs containing human AGT gene with nucleoside A at -217 as compared to the reporter constructs containing nucleoside G at -217**

Since C/EBPβ and IL-6 play an important role in liver specific gene expression, we were
interested in analyzing their effect on the expression of human AGT gene containing A/G polymorphic site at -217. Promoter activity of reporter constructs pHAGT1.3\textit{luc} (with either nucleoside A or G at -217) was determined after IL-6 treatment alone or in the presence of over-expressed C/EBP\textbeta by transient transfection in HepG2 cells. We also compared the promoter activity of these constructs in the presence of over-expressed C/EBP\textbeta in the absence of IL-6 treatment. Results of these experiments (Fig. 6) show that all of these treatments increased the promoter activity of reporter construct pHAGT1.3\textit{luc} (-217A) as well as pHAGT1.3\textit{luc} (-217G). Moreover, promoter activity of the variant A was always greater than the G variant in each experiment. Thus, IL-6 treatment of transfected HepG2 cells increased the overall promoter activity of the variant A by 50% as compared to the G variant (compare panels 1 and 3 of Fig. 6A). Co-transfection of C/EBP\textbeta increased the overall promoter activity of the variant A by 26% as compared to the G variant (compare panels 1 and 3 of Fig. 6B). Co-transfection of C/EBP\textbeta followed by IL-6 treatment increased the overall promoter activity of the variant A by 50% as compared to the G variant (compare panels 1 and 3 of Fig. 6C). We next compared the fold-increase in the promoter activity of each variant with respect to its basal promoter activity. These values are shown over the bars of each pair of reporter construct. IL-6 treatment increased the promoter activity of the A variant by 2.66 fold and of the G variant by 2.18 fold; co-transfection of C/EBP\textbeta increased the promoter activity of A as well as G variant by 1.6 fold; and co-transfection of C/EBP\textbeta followed by IL-6 treatment increased the promoter activity of the A variant by 3.54 fold and of the G variant by 3.03 fold. Results of this experiment suggest that IL-6 treatment of HepG2 cells preferentially enhances the promoter activity of the -217A variant of human AGT gene, particularly in
the case of over-expressed C/EBPβ.

We also studied the effect of over-expressed C/EBPβ and/or IL-6 treatment on the promoter activity of 5'-deleted reporter construct pHAG303luc containing either nucleoside A or G at -217. Results of this experiment (Fig.7) also show that all of these treatments increased the over-all promoter activity of both of the variants. In addition, promoter activity of the variant A was always greater than the G variant in each experimental condition. We also compared fold-increase in the promoter activity of each variant with respect to its basal promoter activity. These values are shown over the bars of each pair of reporter construct. IL-6 treatment increased the promoter activity of the A variant by 3.9 fold and of the G variant by 3.7 fold; co-transfection of C/EBPβ increased the promoter activity of the A variant by 3.6 fold and of the G variant by 4.2 fold; and co-transfection of C/EBPβ followed by IL-6 treatment increased the promoter activity of the A variant by 5.7 fold and of the G variant by 5.5 fold. Results of this experiment also suggest that IL-6 treatment preferentially increases the promoter activity of the A variant, especially in the presence of C/EBPβ. Since the fold-increase in the promoter activity of variants A was greater in pHAGT1.3luc as compared to pHAGT303luc, it would suggest that nucleotide sequence in up-stream region of the promoter also plays a role in IL-6 induced expression of this gene.
Discussion

To date, the AGT gene locus is the only locus that has been associated with human essential hypertension. We have presented evidence that an A/G polymorphism at -217 may be involved in hypertension in the African-American population. The frequency of the -217A allele was significantly increased in African-American hypertensive subjects as compared to normotensive controls. On the other hand, the frequency of -217A allele was not significantly different in Caucasian hypertensive and normotensive subjects. We have also found that 65% of African-American normotensive controls were GG homozygotes whereas 48% of African-American hypertensive subjects were GG homozygotes ($p = .0021$). This observation suggests that -217G allele may be partially responsible for protection of African-American subjects from hypertension. On the other hand 80% of normotensive Caucasian controls were GG homozygotes and 72% of Caucasian hypertensive subjects were GG homozygotes, which is not significantly different ($p = .14$). In accordance with previous studies, we also found that although the frequency of -6A allele is increased in African-American subjects, this difference is not significant between hypertensive and normotensive subjects.

In order to understand the biological significance of this polymorphic site, we constructed three types of reporter constructs containing either nucleoside A or G at -217 and used these reporter constructs in transient transfection assays in human liver derived HepG2 cells. Transient transfection of these reporter constructs indicated statistically significant increased basal promoter activity of reporter constructs containing nucleoside A at -217 as compared to reporter constructs containing nucleoside G at -217.
The nucleotide sequence of AGT gene promoter containing A/G polymorphic site at -217 has partial homology with the binding site of C/EBP family of transcription factors. Our gel shift assays have shown that recombinant C/EBPα, C/EBPβ, and DBP bind more strongly to an oligonucleotide containing human AGT gene promoter with nucleoside A at -217 compared to the same oligonucleotide containing nucleoside G at -217. Since C/EBP family of transcription factors play an important role in IL-6 induced expression of a number of genes, we studied the effect of IL-6 and C/EBPβ on the promoter activity of reporter constructs containing either -217A or -217G by transient transfection in HepG2 cells. Results of our experiments have shown that over-expression of C/EBPβ in the presence or absence of IL-6, or IL-6 treatment alone increases the overall promoter activity of reporter constructs containing the -217A variant as compared to the -217G variant. In addition, our data show that treatment of cells with IL-6 enhances the promoter activity of the -217A variant as compared to the -217 variant, particularly in the case of over-expressed C/EBPβ. Since IL-6 and C/EBPβ enhance the expression of human AGT gene together, our data suggest that modification of C/EBPβ or another interacting factor by IL-6 is involved in selective up-regulation of the -217A variant of this gene.

AGT gene is primarily expressed in liver and adipose tissue and the C/EBP family of transcription factors play a crucial role in regulating expression of a number of genes in these tissues. C/EBPs are a family of leucine zipper transcription factors involved in the regulation of various aspects of cellular differentiation and function(28,29). Six different members of this family have been identified, all sharing a strong homology in the
carboxyl-terminal region (that carries a basic DNA binding domain) and a leucine zipper motif (30-32). The leucine zipper is a heptad of leucine repeats that intercalate with repeats of the dimerization partner forming a coil of α-helices in parallel orientation(33,34). This dimerization is essential for binding of the C/EBP family of transcription factors to cis-acting DNA elements.

AGT is an acute phase protein and its expression is increased by LPS, IL-6, and glucocorticoid treatment(35-38). An acute phase response unit (APRU) located between -470 and -554 has been identified in the rat AGT gene (39). This region of the promoter contains a composite NF-κB and C/EBP binding site located between -531 and -557, one full GRE located between -570 and -584 and a half GRE located between -470 and -477. All of these sites are required for maximum acute phase response of this gene. Although, expression of both rat and human AGT genes is increased in response to acute phase reaction, the APRU observed in the rat gene promoter is absent in the human gene promoter. Similarly, nucleotide sequence around the A/G polymorphic site at -217 of the human AGT gene is not conserved in the rat gene. We have previously shown that nucleotide sequence located between -99 and -91 of the human AGT gene binds to the C/EBP family of transcription factors and this region of the promoter plays an important role in DBP and C/EBPβ induced expression of this gene(23). We have also shown that CREB binds to the nucleotide sequence located between -840 and -830 of the human AGT gene and this sequence is involved in cAMP induced expression of the human AGT gene(40). It has been shown previously that human AGT gene has a C/A polymorphic site at -20 (located between TATA box and transcriptional initiation site). We have
shown that USF binds to this sequence when nucleoside C is present at -20 and ER binds to this sequence when nucleoside A is present at -20(41). Orphan receptor Arp-1 also binds to this sequence and reduces ER induced promoter activity(42). Yanai et al., have shown that the nucleotide sequence located between the TATA box and transcriptional initiation site of the human AGT gene binds to USF and plays a critical role in its expression(43). They have also shown that the liver enriched transcription factor HNF4 binds to the human AGT gene promoter and regulates expression of this gene in hepatocytes(44). In addition, we have shown that the liver enriched transcription factor HNF-3 binds to the nucleotide sequence located between +10 and +20 of the human AGT gene promoter(45). All of these transcription factors including C/EBP (that differentially binds to A/G polymorphic site at -217) may interact with transcriptional co-activator CBP and co-ordinately regulate the expression of this gene.

In conclusion, our data suggest that an A/G polymorphism at -217 of the human AGT gene (that affects the binding of C/EBP family of transcription factors and affects the basal promoter activity of the human AGT gene) may be involved in essential hypertension in the African-American population. So far we have only analyzed DNA from members of the African-American population in the New York area and it will be important to extend these studies to African-American populations living in other areas. It is important to mention that hypertension is a complex multigenic disease and other genes may also be involved in the etiology of this disease. Future studies will help us understand mechanism involved in increased expression of the -217A variant of this gene.
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Legends to Figures

1. Genomic DNA from hypertensive patients and normotensive controls was amplified to produce 233 bp fragment as described in Materials and Methods. Nucleotide sequence of the amplified fragment around A/G polymorphic site at -217 of the AGT gene is shown in top line. AluI restriction site (that will cleave the amplified DNA if nucleoside A is present at -217) is shown in second line, HpaII restriction site (that will cleave the amplified DNA if nucleoside G is present at -217) is shown in the third line. The amplified DNA fragments were treated either with AluI (upper panel) or with HpaII (lower panel) and separated by 3.5% agarose gel. Lane 1 shows the position of DNA markers. Lanes 2-5 show DNA samples from AA homozygotes, lanes 6-8 show DNA samples from A/G heterozygotes, lanes 9-12 show DNA samples from GG homozygotes.

2. Basal promoter activity of reporter constructs containing nucleoside A or G at -217 of the human AGT gene promoter. Reporter construct pHAGT1.3luc, pHAGT303luc, or (223)2luc (with either nucleoside A or G at -217) was transiently transfected in HepG2 cells in six well plates as described in Experimental procedures. Cell extracts were prepared after 48 h of transfection, and luciferase and β-gal activity were measured as described. Luciferase activity was normalized with the β-gal activity. Panel A shows the luciferase activity of pHAGT1.3luc, panel B shows the luciferase activity of pHAGT303luc and panel C shows the luciferase activity of (223)2luc. Empty bars show the promoter activity of reporter constructs containing nucleoside G at -217 and filled
bars show the promoter activity of reporter constructs containing nucleoside A at -217. Promoter activity of each reporter construct containing nucleoside A at -217 was calculated by assuming the promoter activity of same reporter construct containing nucleoside G at -217 as one.

3. (A) The top line shows the nucleotide sequence of consensus C/EBP binding site (TT/GNNCAAT/G) in opposite orientation, the second line shows the nucleotide sequence located between -217 and -225 of the human AGT gene with nucleoside A at -217, and the third line shows the same sequence with nucleoside G at -217 (mismatched nucleosides are marked by asterisks). (B) Electrophoretic mobility shift assay of oligonucleotides 223A and 223G in the presence of recombinant C/EBPα. Lane 1 shows the gel shift assay in the presence of r-C/EBPα alone, lane 2 shows the assay in the presence of a 100 fold excess of cold oligonucleotide 223A, lane 3 shows the assay in the presence of a non-specific cold oligonucleotide containing consensus NF-1 binding site, lane 4 shows the assay in the presence of C/EBPα antibody, and lane 5 shows the assay in the presence of pre immune serum. Lanes 6-10 show the gel shift assay described above in the presence of radiolabeled oligonucleotide 223G.

4. Electrophoretic mobility shift assay of oligonucleotides 223A and 223G in the presence of recombinant C/EBPβ. Lane 1 shows the assay with oligonucleotide 223A and recombinant C/EBP-β, and lane 2 shows the same assay in the presence of C/EBPβ antibody. Lane 3 shows the assay with an oligonucleotide containing consensus C/EBP binding site and recombinant C/EBP-β, and lane 4 shows the same reaction in the
presence of C/EBPβ antibody. Lanes 5,6 show the gel shift assay using 20,000 cpm of purified oligonucleotide 223A in the presence of 2 and 4 µl of r-C/EBPα; lanes 7,8 show the assay using 20,000 cpm of purified oligonucleotide 223G in the presence of 2 and 4 µl of r-C/EBPα. Lanes 9,10 show the reaction using 20,000 cpm of purified oligonucleotide 223A in the presence of 2 and 4 µl of r-C/EBPβ; lanes 11,12 show the reaction using 20,000 cpm of purified oligonucleotide 223G in the presence of 2 and 4 µl of r-C/EBPβ.

5. Electrophoretic mobility shift assay of oligonucleotides 223A and 223G in the presence of recombinant DBP. Lanes 1 and 2 show the gel shift assay using 20,000 cpm of purified oligonucleotide 223A or 223G in the presence of equal amount of r-DBP. Lane 3 shows the gel shift assay using oligonucleotide 223A and r-DBP in the presence of DBP antibody, lane 4 shows the same assay in the presence of a non-specific NF-1 antibody, and lane 5 shows the same assay in the presence of a 100 fold excess of an oligonucleotide containing consensus C/EBP binding site. Lanes 6 and 7 show the gel shift assay of oligonucleotide 223A in the presence of a 100 fold excess of cold oligonucleotides containing HNF3 and NF-1 consensus binding sites respectively. The supershifted band in lane 3 is shown by a broken arrow.

6. Effect of co-transfection of C/EBPβ and/or IL-6 treatment on the promoter activity of reporter constructs pHAGT1.3luc containing either nucleoside A or G at -217. Reporter construct was transfected either alone or with the expression vector MSV C/EBPβ in HepG2 cells as described previously. After 24h of transfection, one group of cells were
treated with recombinant human IL-6 (10 ng/ml) for 24h and promoter activity was analyzed. Section A shows the effect of IL-6, section B shows the effect of co-transfected C/EBPβ, and section C shows the effect of IL-6 and co-transfected C/EBPβ on the promoter activity. Empty bars (panel 4) show the basal promoter activity of the G variant, slanted bars (panel 2) show the basal promoter activity of the A variant, crossed bars (panel 3) show the promoter activity of variant G under experimental condition, horizontal bars (panel 1) show the promoter activity of variant A under experimental condition. Promoter activity of each reporter construct was calculated by assuming the basal promoter activity of pHAGT1.3luc(-217G) as one. All experiments were conducted in sextuplicate in four independent transfections.

7. Effect of co-transfection of C/EBPβ and/or IL-6 treatment on the promoter activity of reporter constructs pHAGT303luc containing either nucleoside A or G at -217. Experimental conditions were same as described in Fig.6. Promoter activity of each reporter construct was calculated by assuming the promoter activity of pHAGT303luc(-217G) as one.
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|                                      | A allele | G allele | p value  |
|--------------------------------------|----------|----------|----------|
| **African-American**                 |          |          |          |
| Hypertensive (n=186)                 | 0.29     | 0.71     | p=0.0017 |
|                                      |          |          | OR= 1.792|
| Normotensive (n=156)                 | 0.19     | 0.81     |          |
| **Caucasian**                        |          |          |          |
| Hypertensive (n=127)                 | 0.15     | 0.85     | p=0.1208 |
|                                      |          |          | OR=1.507 |
| Normotensive (n=135)                 | 0.11     | 0.89     |          |
### Table-II

**Statistical Analysis of -217A/G Polymorphism of Angiotensinogen Gene Based on the Genotype Distribution Using A Allele Dominant Model**

|                      | (AA + AG) | GG  | p value | OR     |
|----------------------|-----------|-----|---------|--------|
| **African-American** |           |     |         |        |
| Hypertensive (n=186) | 12        | 84  | 90      | p=0.0021 OR=2.015 |
| Normotensive (n=156) | 4         | 50  | 102     |        |
| **Caucasian**        |           |     |         |        |
| Hypertensive (n=127) | 4         | 31  | 92      | p=0.1433 OR=1.595 |
| Normotensive (n=135) | 3         | 23  | 109     |        |
### Table-III

**Statistical Analysis of -6A/G Polymorphism of Human Angiotensinogen Gene Based on the Allele Frequency**

|                        | A     | G     | p         | Odds ratio |
|------------------------|-------|-------|-----------|------------|
| **Caucansian**         |       |       |           |            |
| Hypertensive (n=127)   | 0.55  | 0.45  | 0.0670    | 1.383      |
| Normotensive (n=135)   | 0.47  | 0.53  |           |            |
| **African-American**   |       |       |           |            |
| Hypertensive (n=186)   | 0.868 | 0.1317| 0.5800    | 1.140      |
| Normotensive (n=156)   | 0.853 | 0.147 |           |            |
Analysis of genomic DNA for A/G polymorphism at -217 of the angiotensinogen gene

Amplified sequence: CCCTGCACC A/G GCTCACTCT
Alu I site: A GCT
Hpa II site: CC G G

Fig. 1
Basal Promotor activity of reporter construct pHAG1.3 luc, pHAG303 luc or (223) 2 luc containing nucleoside A at -217 is increased on transient transfection in HepG2 cells as compared to the same reporter construct containing nucleoside G at -217.

Fig. 2

Relative Luciferase Activity

|   | A | B   | C |
|---|---|-----|---|
| 0 |   | 1.0 |   |
| 1 |   | 1.2 |   |
| 2 |   | 1.6 |   |
| 3 |   | 2.0 |   |

Fig. 2
Sequence homology between -217 region of the human angiotensinogen gene with C/EBP site

C T T G C N N C A

C C T G C A C C A

*    *    *    *

C C T G C A C C G

*    *    *

An oligonucleotide containing nucleoside A at -217 binds strongly to the recombinant C/EBP-α

--- Recombinant C/EBP-alpha ---

|   | 223A | 223G |
|---|------|------|
| Cold 223A/G | - | + | - | - | - | + | - | - | - |
| Cold NF-1 | - | - | + | - | - | - | + | - |
| C/EBPab | - | - | + | - | - | - | - | + |
| PIS | - | - | - | + | - | - | - | - | + |

Fig. 3
Oligonucleotide 223A binds strongly to recombinant C/EBP-β as compared to the oligonucleotide 223G

|                | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|----------------|---|---|---|---|---|---|---|---|---|----|----|----|
| C/EBP beta     | + | + | + | + | - | - | - | + | + | +  | +  | +  |
| C/EBP alpha    | - | - | - | - | + | + | + | - | - | -  | -  | -  |
| C/EBP beta ab  | - | + | - | + | - | - | - | - | - | -  | -  | -  |
| 223A oligo     | + | + | - | - | + | + | - | - | + | +  | -  | -  |
| 223G oligo     | - | - | - | - | - | + | + | - | - | +  | +  | +  |
| C/EBP oligo    | - | - | + | + | - | - | - | - | - | -  | -  | -  |

Fig. 4
Oligonucleotide 223A binds strongly to recombinant DBP as compared to the oligonucleotide 223G.

|               | Recombinant DBP |
|---------------|-----------------|
| 223A          | + - + + + + +   |
| 223G          | - + - - - - -   |
| DBP ab        | - - + - - - -   |
| NF-1 ab       | - - - + - - -   |
| Cold C/EBP oligo | - - - + - -   |
| Cold HNF3 oligo     | - - - - - + - |
| Cold NF-1 oligo       | - - - - - - + |

Fig. 5

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IL-6 treatment in the presence or absence of C/EBPβ increases the promoter activity of pHAG1.3\textit{lac}(-217A) as compared to pHAG1.3\textit{lac} (-217G)

![Graph showing relative luciferase activity](image-url)

**Fig. 6**

Relative Luciferase Activity

- A: x2.66
- B: x2.18
- C: x1.62
- D: x1.59

Values are given as mean ± S.E.M.
IL-6 treatment in the presence or absence of C/EBP β increases the promoter activity of pHAG303luc(-217A) as compared to pHAG303luc(-217G).

Fig. 7

Relative Luciferase Activity

|    |   |   |   |   |   |   |   |
|----|---|---|---|---|---|---|---|
| 1  |   |   |   |   |   |   |   |
| 2  |   |   |   |   |   |   |   |
| 3  |   |   |   |   |   |   |   |
| 4  |   |   |   |   |   |   |   |

A: x3.9  x3.7  B: x3.6  x4.27  C: x5.7  x5.5

Fig. 7
Angiotensinogen gene polymorphism at -217 affects basal promoter activity and is associated with hypertension in African-Americans
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