Angiotensinogen Gene Core Promoter Variants and Non-Modulating Hypertension

Karl F. Hilgers, Christian Delles, Roland Veelken, Roland E. Schmieder

Abstract—Non-modulation has been suggested as a possible intermediate phenotype defining a subgroup of genetic hypertension. The trait is characterized by an attenuated response of renal blood flow and/or aldosterone to angiotensin (Ang) II. We tested the hypothesis that functional polymorphisms of the core promoter of the angiotensinogen gene are associated with non-modulation. Fifty-six young, white, male, untreated hypertensive patients and 65 age-matched normotensive volunteers were genotyped for 3 known functional variants of the angiotensinogen core promoter. All subjects were infused with 2 doses (0.5 and 3 ng/kg per minute) of Ang II while they were on a high sodium diet (250 mmol/d). The blood pressure, renal plasma flow, and aldosterone responses to Ang II were not affected by the −6 G/A polymorphism. The −20 A/C variant had no significant effects on the blood pressure or renal hemodynamic response to Ang II. However, the aldosterone response to both doses of Ang II was significantly decreased in −20 C allele carriers compared with −20 AA homozygotes in a multivariate analysis. The −18 T allele was not detected in our population, and there was a linkage dysequilibrium between −20 C and −6 A: −20 C almost exclusively occurred on the −6 A allele. Haplotype analysis indicated that the −20 C/−6 A haplotype but not the −20 A/−6 A haplotype was associated with a decreased aldosterone response to Ang II. We conclude that the −20 C variant or the −20 C/−6 A haplotype of the angiotensinogen core promoter is associated with a blunted aldosterone response to Ang II and may thus contribute to the non-modulating phenotype. (Hypertension. 2001;38:1250-1254.)

Key Words: angiotensin ■ angiotensinogen ■ aldosterone ■ hypertension, non-modulating

Non-modulation has been described by Shoback, Hollenberg, and colleagues1,2 as an abnormal response of aldosterone release or renal blood flow to angiotensin (Ang) II or to sodium restriction, occurring in a subset of hypertensive patients. Somewhat different definitions have been used, emphasizing either renal blood flow or aldosterone responses and sometimes substituting sodium restriction for Ang II infusion.3,4 There is only a partial concordance between aldosterone and renal blood flow response.5 Some authors argued that non-modulating hypertension is a heritable intermediate phenotype5,6 that may precede the development of hypertension.6 Others have questioned the heritability of renal blood flow responses to Ang II.7

The molecular genetic basis of non-modulating hypertension is not fully understood, but Hopkins et al8 reported an association with the M235T polymorphism of the angiotensinogen gene. This variant is associated with hypertension and elevated plasma angiotensinogen in several populations.9–11 More recent research revealed that the elevation of plasma angiotensinogen is not due to the M235T substitution itself but a linked A for G substitution at position −6 in the core promoter of the gene.12 The −6A variant was reported to be associated with elevated angiotensinogen gene transcription,12 increased angiotensinogen gene expression in uterine spiral arteries,13 elevated plasma angiotensinogen levels, and hypertension.14 Interestingly, Fardella et al15 reported an association with high plasma aldosterone.

In addition, 2 other polymorphisms of the angiotensinogen gene core promoter that may cause altered gene transcription have been described: C for A substitution at position −20 leads to increased angiotensinogen transcription in vitro16 and is associated with hypertension and elevated plasma angiotensinogen.17 The C/T polymorphism at position −18 may affect transcription factor binding in vitro,18 and the C allele was associated with hypertension.19

We tested the hypothesis that these angiotensinogen gene promoter variants are associated with non-modulation. The blood pressure, renal plasma flow (RPF), and aldosterone responses to Ang II were measured in normotensive or never-treated mildly hypertensive white volunteers on a high sodium diet. Because of the higher frequency of non-modulation in males,20 the potential interaction between gender and genotype,4 and the effects of the −20 C/A substitution on estrogen-dependent angiotensinogen gene transcription,16 we investigated only male subjects.
Methods

Study Population

White male students (age, 20 to 40 years) were enrolled as described in detail elsewhere. Subjects were considered hypertensive if the average of all casual blood pressure readings was ≥140 mm Hg systolic or ≥90 mm Hg diastolic. Subjects with cardiovascular disease, secondary or World Health Organization stage III hypertension, or previous treatment for hypertension were excluded. The protocol was approved by our Clinical Investigation Committee. Written informed consent was obtained from each participant. Diet was prescribed daily either by increasing their nutritional intake or by infusion, the participants were requested to ingest orally 13 g sodium chloride daily either by measurement of 24-hour sodium excretion. One week before Ang II infusion, the participants were requested to ingest orally 13 g sodium chloride daily either by measurement of 24-hour sodium excretion. One week before Ang II infusion, the participants were requested to ingest orally 13 g dietary salt per day either by increasing their nutritional intake or by infusion, the participants were requested to ingest orally 13 g dietary salt per day either by increasing their nutritional intake or by using salt tablets to facilitate the detection of the response to exogenous Ang II.

Response to Ang II

At 10:00 AM, blood pressure, Ang II, aldosterone, and RPF were measured in the subjects after they had rested for 1 hour in the supine position. We applied the constant infusion technique to determine RPF (p-aminohippurate clearance) without urinary sampling as previously described. This method depends on reaching a steady state and may overestimate RPF by 20%. However, changes from baseline in each subject are not affected by this potential bias, and a new steady state is reached within 20 minutes. At 12:00 noon, a constant infusion of Ang II (0.5 ng/kg per minute, Clinalpha AG) was given. After 30 minutes, the dose of Ang II was increased to 3.0 ng/kg per minute for another 30 minutes. Blood pressure, serum aldosterone, and RPF were measured at the end of both infusion periods. Aldosterone and Ang II were measured as described previously.

Genotyping

From genomic DNA, a 345-bp fragment of the human angiotensinogen gene, starting 222 bp upstream from the transcription start site, was amplified by using the primers 5′-TGCAACGCGTCACTCTGTTCA-3′ and 5′-ATCTCCCGGGCCTTTCTCTCCTA-3′. Thirty-five polymerase chain reaction cycles (94°C, 60°C, and 72°C, each for 1 minute) were performed with a thermocycler (Hybaid Ltd). Figure 1 shows the core promoter sequence containing the polymorphisms and the enzymes used for genotyping. After restriction digestion, gel electrophoresis was performed to determine cleavage of polymerase chain reaction products by the respective enzyme.

Statistical Analysis

Data were analyzed in a multivariate/repeated-measures model with 1 within-subject factor (dose) at 3 levels (Ang II at 0, 0.5, and 3 ng/kg per minute) and 3 between-subject factors: blood pressure status at 2 levels (hypertensive or normotensive), the −6 G/A polymorphism at 3 levels (GG, GA, and AA), and the −20 A/C polymorphism at 2 levels (AA versus AC and CC combined). In a secondary analysis, genotypes based on haplotypes were tested in the same multivariate model (see Figure 1 and Results for details). Analysis was carried out by using SPSS 9.01 software (SPSS Inc). A value of P ≤ 0.05 was considered significant. Values are given as mean ± SD unless indicated otherwise.

Results

The frequency of the −20 C allele was 0.18 in hypertensive patients and 0.14 in normotensive subjects, and the frequency of the −6 A allele was 0.43 in hypertensive patients and 0.47 in normotensive subjects. The −18 T variant was not found in any subject; in 2 of 121 participants, apparent heterozygosity for −18 T/C in one test turned out to be due to an incomplete BssSI restriction digest. Repeated control tests identified both subjects as −18 CC. The −20 A/C and −6 G/A polymorphisms were not associated with differences in age, body mass, ambulatory blood pressure, baseline plasma Ang II, and serum or urine aldosterone (Table 1).

In the multivariate analysis of the response to Ang II, the within-subject factor (Ang II dose) emerged as highly significant (P < 0.001) for all 3 parameters (blood pressure, RPF, and aldosterone), as expected. The blood pressure response was significantly affected by the hypertension status (P < 0.001) but not by the −20 A/C (P = 0.278) and −6 G/A (P = 0.623) polymorphisms (Table 2). The RPF response was also affected by the blood pressure status (P = 0.006) but not by the −20 A/C (P = 0.158) and −6 G/A (P = 0.876) polymorphisms (Table 2). The aldosterone response was not affected by blood pressure status (P = 0.284) or the −6 G/A polymorphism (P = 0.392) but was significantly lower in −20 C allele carriers (P = 0.01, Figure 2).

Because the −20 C allele occurred only in −6 A allele carriers, we also analyzed the aldosterone response by haplotypes. Of 8 possible haplotypes, 4 did not occur because of the absence of the −18 T allele (Figure 1). Thus, the haplotypes could be directly inferred in all subjects homozygous at either the −20 or the −6 locus (105 of 121 participants). The remaining 16 subjects could theoretically carry either haplotypes I and VI or haplotypes II and V.

<table>
<thead>
<tr>
<th>TABLE 1. Clinical Data and Hormone Measurements of All Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameters</td>
</tr>
<tr>
<td>Total subjects, n</td>
</tr>
<tr>
<td>Hypertensive</td>
</tr>
<tr>
<td>Normotensive</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>Body mass index</td>
</tr>
<tr>
<td>24-h systolic BP, mm Hg</td>
</tr>
<tr>
<td>24-h diastolic BP, mm Hg</td>
</tr>
<tr>
<td>Sodium excretion, mmol/24 h</td>
</tr>
<tr>
<td>Plasma Ang II, pmol/mL</td>
</tr>
<tr>
<td>Urinary aldosterone, μg/24 h</td>
</tr>
</tbody>
</table>

BP indicates blood pressure. Data are mean ± SD. There were no significant differences between genotypes.
We assumed that all these 16 subjects carried haplotypes I and IV because of the rarity of haplotype V. Of 44 subjects homozygous for either −20 CC or −6 GG, among whom haplotype V could have been identified unambiguously, only 1 subject carried I haplotype V allele. The estimated haplotype frequencies (Figure 1) would be only marginally affected if our assumption proved to be erroneous.

The size of our population did not permit a full analysis of all genotypes that resulted from the occurring combinations of haplotypes. However, we tested homozygotes for the most frequent “normal” haplotype I (−20 A and −6 G, n=37) against either haplotype II (−20 A and −6 A) carriers (II heterozygotes and II/II homozygotes, n=43) or haplotype VI (−20 C and −6 A) carriers (VI heterozygotes and VI/VI homozygotes, n=19) in the multivariate model. The aldosterone response was affected by haplotypes (P=0.050) but not by blood pressure status (P=0.479). Post hoc testing (Dunnett) showed lower aldosterone response in haplotype VI carriers (see Figure 3) versus both haplotype II carriers (P=0.032) and haplotype I homozygotes (P=0.043), whereas there was no difference between haplotypes I and II (P=1.000).

**Discussion**

Our data support the notion that angiotensinogen gene core promoter variants that lead to increased gene transcription are associated with a blunted aldosterone response to Ang II infusion. Regarding aldosterone response, our results thus indirectly support the concept of Williams, Hopkins, and colleagues, who reported that increased adrenal angiotensinogen may contribute to the non-modulating phenotype in hypertension. We wish to point out that we did not intend to perform a classic association study between some genetic variant and a binary cardiovascular phenotype (eg, hypertension: yes/no) but that the present study was hypothesis-driven: We selected 3 genetic variants of the angiotensinogen gene promoter for which in vivo and/or in vitro evidence that the variant leads to increased angiotensinogen gene transcription had been reported. We compared the aldosterone and renal blood flow response to Ang II in these subjects, stratified for genotypes. We avoided arbitrary assumptions and cutoff values (eg, non-modulation: yes/no) but compared the phenotypic results of the genotype-defined groups according to predefined hypotheses.

At first glance, we obtained the most conclusive set of results in subjects carrying the −20 C allele of the angiotensinogen gene promoter. Because of the low allele frequency, a separate analysis of −20 CC homozygotes (only 3 hypertensive patients and 1 normotensive participant) was not possible. However, −20 C allele carriers clearly had a blunted aldosterone response to Ang II at both doses of the peptide. The data are more difficult to interpret with regard to the −6 G/A polymorphism. On the one hand, none of the responses to Ang II was significantly affected by the −6 A allele. One the other hand, the effects of the −20 A/C and −6 G/A polymorphisms cannot easily be separated because of the linkage disequilibrium between these 2 variants. The −20 C allele occurred almost exclusively in conjunction with −6 A. Only 1 of 121 participants who carried one −20 C/−6 G (haplotype V) allele was identified. All remaining −20 C alleles probably occurred together with −6 A (although some minor uncertainty remains in the 16 subjects heterozygous at both the −20 and the −6 loci).

Our limited analysis of the haplotypes indicates that haplotype II (−20 A/−6 A) has no effect on the aldosterone response but that haplotype VI (−20 C/−6 A) leads to decreased aldosterone release in response to Ang II. This finding suggests a more important role of the −20 A/C than of the −6 G/A polymorphism but remains inconclusive as long as the effects of haplotype V (−20 C/−6 G) cannot be tested. However, such a test proved impossible because of the scarcity of haplotype V. In the absence of further data on haplotype V, we can only conclude that haplotype VI (−20 C/−6 A) is associated with a reduced aldosterone release in response to Ang II. Because both the −6A and the −20 C allele were associated with higher angiotensinogen transcription, our conclusion is in keeping with the notion that variants causing higher angiotensinogen transcription are associated with a blunted aldosterone release.
We did not detect differences according to genotypes in the renal hemodynamic response to Ang II. This observation was unexpected because the settings of the present study (high salt intake) were designed to facilitate detection of differences in renal blood flow responses to Ang II. p-Aminohippurate clearance may be insufficiently exact for the detection of subtle interindividual differences. Furthermore, the method that we used to calculate RPF is dependent on reaching a steady state. Although the 30-minute period for each dose of Ang II should be sufficient to reach a new steady state,24 we cannot exclude the possibility that other techniques (use of radiolabeled tracers or urine sampling) might have been better to detect differences in the response to Ang II. Alternatively, angiotensinogen core promoter variants (or local angiotensinogen synthesis) may be more important in the control of aldosterone release than in the regulation of renal perfusion.3,7 Aldosterone responses have often been studied with subjects on a low salt diet; compared with our high salt conditions, a low salt diet would be expected to emphasize the blunted response in non-modulators, who would not increase their response, in contrast to modulators.1

There are several further limitations of the present study. We studied young white males with only mild hypertension who showed no signs of target organ damage and were on a high sodium diet. Therefore, we do not know whether or not the results can be extended to other populations, eg, older patients with target organ injury. However, we believe that the use of a fairly homogeneous population is important to limit confounding sources of variation in the response to Ang II. The allele frequencies measured by us are not a valid result for an association of angiotensinogen core promoter variants

**Figure 1.** The core promoter of the angiotensinogen gene, the endonucleases used for genotyping, and the haplotypes resulting from the polymorphisms. In the top panel, in the nucleotide sequence of the angiotensinogen core promoter between the TATA box and exon 1, the 3 polymorphisms are highlighted, and the substitutions leading to increased gene transcriptions are shown above the sequence. The numbers indicate the distance in nucleotides from the transcription start site. The recognition sequences and actions of the restriction endonucleases are shown. The polymorphisms are highlighted, and the substitutions leading to a lack of cleavage by the respective enzyme are shown below the sequence. The bottom panel shows the 8 possible haplotypes and the estimated haplotype frequencies in the study population (see text for details).

We did not detect differences according to genotypes in the renal hemodynamic response to Ang II. This observation was unexpected because the settings of the present study (high salt intake) were designed to facilitate detection of differences in renal blood flow responses to Ang II. p-Aminohippurate clearance may be insufficiently exact for the detection of subtle interindividual differences. Furthermore, the method that we used to calculate RPF is dependent on reaching a steady state. Although the 30-minute period for each dose of Ang II should be sufficient to reach a new steady state,24 we cannot exclude the possibility that other techniques (use of radiolabeled tracers or urine sampling) might have been better to detect differences in the response to Ang II. Alternatively, angiotensinogen core promoter variants (or local angiotensinogen synthesis) may be more important in the control of aldosterone release than in the regulation of renal perfusion.3,7 Aldosterone responses have often been studied with subjects on a low salt diet; compared with our high salt conditions, a low salt diet would be expected to emphasize the blunted response in non-modulators, who would not increase their response, in contrast to modulators.1

There are several further limitations of the present study. We studied young white males with only mild hypertension who showed no signs of target organ damage and were on a high sodium diet. Therefore, we do not know whether or not the results can be extended to other populations, eg, older patients with target organ injury. However, we believe that the use of a fairly homogeneous population is important to limit confounding sources of variation in the response to Ang II. The allele frequencies measured by us are not a valid result for an association of angiotensinogen core promoter variants

**Figure 2.** Serum aldosterone at baseline and after 2 doses of Ang II in subjects grouped for the genotype at the −6 G/A polymorphism (A) or grouped for the −20 A/C polymorphism (B). Data are given as mean±SEM. *Significant (P<0.05) differences between genotypes (see text for details of statistical analysis).

**Figure 3.** Serum aldosterone at baseline and after 2 doses of Ang II in subjects homozygous for the most frequent haplotype I (−20 A, −6 G) compared with haplotype II carriers (−20 A, −6 A; I/II heterozygotes and II/II homozygotes combined) and haplotype VI carriers (−20 C, −6 A; I/VI heterozygotes and VI/VI homozygotes combined). Data are given as mean±SEM. $P=0.043$ between I/I and I/VI+VI/VI; $\delta P=0.032$ between I/II+I/II and I/VI+VI/VI (see text for details of statistical analysis).
with hypertension, because we recruited volunteers and excluded patients with more severe hypertension or signs of organ damage. Nevertheless, the allele frequencies for all 3 polymorphisms were within the ranges found in population-based samples. The frequency of the −20 C allele in the present study, 0.157, was lower than the frequency of 0.266 reported in a Japanese population but close to the frequency of 0.15 reported from Utah and the frequency of 0.19 reported in a white population from England. The frequency of the −6 A allele was 0.45 in our sample and 0.44 in a large white population (1509 subjects). The absence of the T for C substitution at position −18 in our sample is in agreement with results from Japanese and English populations. Even Sato et al. who reported an association with hypertension, described a very low frequency of 0.027 for the −18 T allele. The reason for this difference from the initially reported −18 T allele frequency of 0.13 to 0.19 remains unclear.

Although we did not find the −18 T variant, our results do support the notion that angiotensinogen gene core promoter polymorphisms play a role at least for some forms of hypertension. In particular, our data draw attention to the −20 A/C polymorphism. Evidence has been provided that this polymorphism affects transcription factor binding and the gene transcription rate. The −20 C allele is associated with hypertension and elevated plasma angiotensinogen in a Japanese population but has hitherto only rarely been studied in other populations. The linkage disequilibrium between −6 A and −20 C raises the possibility that some previous findings ascribed to the −6 A variant might in fact have been due to the overrepresentation of −20 C in −6 A allele carriers. The limited analysis of haplotypes that our data permit suggests an especially important role of the −20 C/−6 A haplotype for non-modulating hypertension.

Acknowledgments

This study was supported by grants-in-aid by the Deutsche Forschungsgemeinschaft to Dr Hilgers (Hi 510/6-1) and Dr Schmieder (Schm 638/8-2). We acknowledge the technical expert assistance of Ortrun Alter and Anja Friedrich.

References


Angiotensinogen Gene Core Promoter Variants and Non-Modulating Hypertension
Karl F. Hilgers, Christian Delles, Roland Veelken and Roland E. Schmieder

Hypertension. 2001;38:1250-1254
doi: 10.1161/hy1201.096545

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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/38/6/1250

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/