Structural Analysis and Evaluation of the Aldosterone Synthase Gene in Hypertension

Eva Brand, Nathalie Chatelain, Paolo Mulatero, Isabelle Féry, Kathleen Curnow, Xavier Jeunemaitre, Pierre Corvol, Leigh Pascoe, Florent Soubrier

Abstract—Anomalies in either of the tightly linked genes encoding the enzymes CYP11B1 (11β-hydroxylase) or CYP11B2 (aldosterone synthase) can lead to important changes in arterial pressure and are responsible for several monogenically inherited forms of hypertension. Mutations in these genes or their regulatory regions could thus contribute to genetic variation in susceptibility to essential hypertension. To test this hypothesis, we performed 2 complementary studies of the CYP11B1/CYP11B2 locus in essential hypertension. After characterizing a DNA contig containing the CYP11B1 gene and mapping the gene in the Centre d’Etudes du Polymorphisme Humain reference panel of families, we performed a linkage study with 292 hypertensive sibling pairs and a highly informative microsatellite marker near CYP11B1. We also analyzed the association of 2 frequent biallelic polymorphisms of the CYP11B2 gene, in the promoter at position −344 (−344C/T) and the other, a common gene conversion in intron 2, with hypertension in 380 hypertensive patients and 293 normotensive individuals. Statistical analyses did not show significant linkage of the CYP11B1 microsatellite marker to hypertension. No positive association with hypertension was found with the gene conversion in intron 2, but a positive association with hypertension was found with the −344T allele. The hypertensive and normotensive samples differed significantly in both genotype (P=0.023) and allele frequencies (P=0.010). Our data suggest a modest contribution of the CYP11B2 gene to essential hypertension. (Hypertension. 1998;32:198-204.)

Key Words: aldosterone synthase □ steroid 11β-hydroxylase □ biallelic polymorphism □ microsatellite marker □ association study □ linkage study

The cytochrome P450, CYP11B1, a steroid 11β-hydroxylase, catalyzes the terminal step of cortisol biosynthesis.1,2 The enzyme is expressed at high levels throughout the human adrenal cortex and is positively regulated by corticotropin.3 A related enzyme, CYP11B2 (aldosterone synthase), also has steroid 11β-hydroxylase activity as well as the 18-hydroxylase and 18-oxidase activities required for the terminal steps of aldosterone biosynthesis.1,2 Expression of this enzyme is limited to the adrenal zona glomerulosa, where it is principally regulated by serum levels of potassium and angiotensin II.4 The two genes encoding these enzymes are located on chromosome 8q22 ~40 kb apart.4-6 Each gene contains 9 exons and extends over >8000 bp of DNA.7 The nucleotide sequences of these genes are 95% identical in coding regions and ~90% identical in introns, whereas the encoded proteins are 93% identical in their predicted amino acid sequences.

Mutations in CYP11B1 lead to a hypertensive form of congenital adrenal hyperplasia due to the accumulation of 11-deoxycorticosterone and its metabolites, which have mineralocorticoid activity. In contrast, mutations in CYP11B2 lead to various forms of aldosterone synthase deficiency, characterized by salt wasting and hypotension.11,12 Furthermore, unequal recombination between the 2 genes, leading to a duplicated hybrid CYP11B gene, causes the dominantly inherited hypertensive disorder of glucocorticoid-suppressible hyperaldosteronism.5 In this disease, the hybrid gene encodes an enzyme with aldosterone synthase activity, which is expressed throughout the adrenal cortex under the control of CYP11B1 regulatory elements. Consequently, aldosterone is improperly synthesized in the zona fasciculata/reticularis of the adrenal cortex under the control of corticotropin.

It is possible that other mutations in these genes or in their regulatory elements could also contribute to the genetic susceptibility to essential hypertension. To test the genetic linkage of these genes to essential hypertension, we carried out a genetic analysis of this chromosomal region in 292 sibling pairs with essential hypertension with the use of affected sib-pair methods and a highly polymorphic dinucleotide repeat identified in a P1 clone containing the CYP11B1 gene.

We also performed a case-control study of 2 common polymorphisms in the CYP11B2 gene. The first polymorphism (−344C/T) involves a C/T substitution in a putative binding site for the steroidogenic transcription factor SF-1; previous studies have shown a 4-fold increase in binding of SF-1 to the −344C allele, which could result in an altered...
transcription rate. The second polymorphism is a common gene conversion in intron 2 of CYP11B2, in which most of the intron is replaced by that of CYP11B1. The association of these two biallelic CYP11B2 polymorphisms was studied in 380 hypertensive individuals and 293 white controls.

Methods

Study Population

This study was approved by a local review committee, and all subjects gave informed consent. A total of 380 hypertensive cases were selected in 3 collaborating centers in France (Paris, Toulouse, and Bordeaux) according to the following criteria: (1) onset of hypertension at <60 years of age; (2) established hypertension as defined by either a diastolic blood pressure DBP >90 mm Hg in treated patients or a DBP >95 mm Hg on 2 consecutive visits for those untreated; (3) the absence of secondary forms of hypertension, as determined by appropriate clinical investigation in the collaborating center; and (4) families with at least 2 siblings affected by hypertension. Subjects with a history of alcohol intake >50 g/24 h, oral contraceptive therapy, diabetes mellitus, or renal impairment were excluded. Blood pressure was measured in the supine position with a sphygmomanometer. Application of these criteria led to the ascertainment of 170 sibships in which 2 or more offspring were treated patients or a DBP >90 mm Hg on 2 consecutive visits for those untreated; (3) the absence of secondary forms of hypertension, as determined by appropriate clinical investigation in the collaborating center; and (4) families with at least 2 siblings affected by hypertension. Subjects with a history of alcohol intake >50 g/24 h, oral contraceptive therapy, diabetes mellitus, or renal impairment were excluded. Blood pressure was measured in the supine position with a sphygmomanometer. Application of these criteria led to the ascertainment of 170 sibships in which 2 or more offspring were hypertensive (125 pairs, 37 trios, 6 quartets, and 2 quintets). A total of 292 affected sibling pairs were thus evaluated.

Genotype Analysis of Dinucleotide Repeat Polymorphism at the Human CYP11B1 Locus

The CYP11B1 dinucleotide repeat genotypes were determined by PCR amplification of genomic DNA with [γ-32P]ATP end-labeled primers (oligonucleotides 8 and 9 of Table 2) flanking the CA repeat and subsequent analysis by denaturing acrylamide gel electrophoresis. Allele identity was checked in all gels by comparison with a control DNA. Population allele frequencies of the CYP11B1 microsatellite were estimated from the families by using the ILINK program. The samples were denatured at 94°C for 5 minutes, followed by 35 amplification cycles.

Genotyping of CYP11B2 Polymorphisms

The genomic region encompassing the biallelic polymorphism (−344/CT) was amplified by using oligonucleotide primers 1 and 2 shown in Table 2. The amplification was performed with 100 ng of DNA in a total volume of 50 μL containing 10 mmol/L Tris HCl (pH 9), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.1% Triton X-100, 0.2 mg/mL BSA, 200 μmol/L dNTPs, 25 pmol of each primer, and 0.2 U Taq polymerase (ATGC Biotechnologie). The samples were denatured at 94°C for 5 minutes, followed by 35 amplification cycles.
at 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds and 1 cycle at 72°C for 10 minutes. After enzymatic amplification, one fifth of the PCR product was denatured in 150 µL of 0.5 mol/L NaOH and 1.5 mol/L NaCl and blotted onto nylon membranes (N+, ICN). Membranes were then neutralized in 2× SSC and cross-linked with UV light. Genotyping was performed by using allele-specific oligonucleotides. Each membrane was hybridized in 7% polyethylene glycol–10% SDS at 50°C for 4 hours with 100 pmol of either of the 2 oligonucleotides (3 and 4, Table 2) end labeled with [γ-32P]ATP. The membranes were washed twice at room temperature in 1× SSC for 5 minutes followed by 5 minutes in 0.5× SSC at 52°C, followed by autoradiography.

Analysis of the intron 2 polymorphism was carried out by allele-specific PCR as follows. The samples were denatured at 94°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute followed by 1 cycle at 72°C for 10 minutes. Amplified products were electrophoresed in a 1.5% agarose gel and visualized by staining with ethidium bromide. Oligonucleotides 5 (localized in intron 3 of CYP11B1 and CYP11B2) and 6 (localized in intron 2 of CYP11B2) detected the nonconverted allele containing the CYP11B2 sequence. Oligonucleotides 5 and 7 (localized in intron 2 of CYP11B1) detected the converted allele, when it was present. DNA from subjects known to carry the normal and converted alleles by sequencing were amplified as positive controls.

Statistical Analysis

The frequencies of the 13 alleles of the microsatellite marker were estimated from all pedigrees collected with the use of the ILINK program of the LINKAGE package (0.001, 0.007, 0.082, 0.298, 0.344, 0.414, 0.408, 0.060, 0.004, 0.003, 0.003, 0.003, and 0.003). The frequency of the −344T allele (0.546) and of the intron 2 conversion (0.5) was evaluated with the same program.

Localization of CYP11B1 Gene and Identification of Flanking Microsatellite Markers

A 60-kb contig of subclones containing the CYP11B1 gene was assembled as set out in the Methods section. A BamHI, EcoRII restriction site map of this contig is presented in Figure 1. A dinucleotide repeat was identified in the 3′-flanking region of the CYP11B1 gene. The entire coding region of the CYP11B1 gene was isolated on a single BamHI fragment of 8.5 kb. The surrounding sequence included a truncated long interspersed repeat element (LINE-1) 400 bp 5′ from the transcription start site, suggesting that the most important promoter elements lie close to the gene as well as another LINE element farther 3′ from the gene. An open reading frame with >95% sequence homology to an expressed sequence tag (EST) from a human brain cDNA library was also found 3′ from the gene in the P1 clone (data not shown).

Results

Hypertensive Sibships by 2 Different Methods (SAGE and ANALYZE)

Table 3. Linkage Analysis With CYP11B1 Microsatellite Marker and 2 Biallelic Polymorphisms of CYP11B2 in Hypertensive Sibships by 2 Different Methods (SAGE and ANALYZE)

In the case-control study of the 2 biallelic markers, comparisons of genotype distributions and of allelic frequencies were assessed by the χ2 test with 2 and 1 df, respectively. Deviation from Hardy-Weinberg equilibrium was tested by the χ2 test with 1 df.

Table 3. Linkage Analysis With CYP11B1 Microsatellite Marker and 2 Biallelic Polymorphisms of CYP11B2 in Hypertensive Sibships by 2 Different Methods (SAGE and ANALYZE)

<table>
<thead>
<tr>
<th>Markers</th>
<th>n</th>
<th>χ2±SE</th>
<th>P</th>
<th>Shared/Unshared</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA repeat</td>
<td>195</td>
<td>0.48±0.02</td>
<td>NS</td>
<td>98.4/116.9</td>
<td>NS</td>
</tr>
<tr>
<td>C −344T</td>
<td>292</td>
<td>0.50±0.01</td>
<td>NS</td>
<td>90.9/97.6</td>
<td>NS</td>
</tr>
<tr>
<td>Conversion in intron 2</td>
<td>248</td>
<td>0.48±0.01</td>
<td>NS</td>
<td>82.0/99.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

n indicates number of sib pairs; and χ2, mean proportion of alleles identical by descent.
markers AFMb014xe1 (D8S1744) and AFMa082wh9 (D8S1836) with odds 1000:1 (Figure 2).

**Linkage Study**

Because parental genotypes were generally not available, allele frequencies were estimated by the ILINK program (LINKAGE) of the total group of families. Results of linkage analysis with the CYP11B1 microsatellite marker and the 2 biallelic polymorphisms of CYP11B2 (−344C/T and intron 2 conversion) in hypertensive sibships are shown in Table 3. No significant excess of shared alleles was observed in the whole panel by using either of the programs for sib-pair analysis; the calculated number of alleles shared was close to 0.5 in each case.

The statistical analysis was also performed on subgroups of families defined according to criteria used in previous studies. These criteria included a body mass index (BMI) ≤27 kg/m², severe hypertension (DBP ≥100 mm Hg or ≥2 antihypertensive treatments), or an early age of onset (<45 years of age). Application of these criteria, isolated or in combination, did not result in any significant evidence for linkage.

**Association Study**

An association study was performed by comparing allele and genotype frequencies for the 2 biallelic polymorphisms of CYP11B2 (−344C/T and intron 2 conversion) in hypertensive patients (n=380 and n=369) and normotensive control subjects (n=293 and n=277). We did not observe significant differences between the 2 groups for the intron 2 conversion. The frequency of the gene conversion in our patients was 0.45 in hypertensives and 0.43 in normal controls ($\chi^2=0.66$, $P=0.41$).

A significant association with hypertension was found, however, with the T allele of the promoter polymorphism of CYP11B2 (−344C/T). The polymorphism −344C/T did not exhibit significant deviation from Hardy-Weinberg expectations in the case or control groups. A significant difference in genotype ($P=0.023$) and allele ($P=0.010$) frequencies was found between cases and controls. We also compared control subjects and the subgroups of hypertensive subjects defined above. The association remained significant in the subgroup with early-onset hypertension ($P=0.005$) but was not significant when BMI was ≤27 kg/m², suggesting heterogeneity in the cause of hypertension in these patients (Table 4).

**Figure 2.** Line graph shows location (log of the odds [lod] score) for placement of the CYP11B1 locus with respect to chromosome 8 markers characterized in the Centre d’Etudes du Polymorphisme Humain (Paris, France) reference families. Maximum likelihood placement (maximum of log of the odds score curve) for CYP11B1 is indicated by the arrow. Odds against alternative orders are also indicated. Distances along the x axis are in centimorgans (cM).
TABLE 4. Comparison of Allele and Genotype Frequencies (Freq) of C–344T Polymorphism in Controls and Hypertensive Cases

<table>
<thead>
<tr>
<th></th>
<th>TT</th>
<th>CT</th>
<th>CC</th>
<th>(\chi^2)</th>
<th>Freq (%)</th>
<th>(\chi^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>71 (24.2%)</td>
<td>144 (49.2%)</td>
<td>78 (26.6%)</td>
<td>4.88</td>
<td>0.561</td>
<td>6.687 P=0.010</td>
</tr>
<tr>
<td>Group 1</td>
<td>116 (30.5%)</td>
<td>194 (51.1%)</td>
<td>70 (18.4%)</td>
<td>7.537 P=0.023</td>
<td>0.561</td>
<td>6.687 P=0.010</td>
</tr>
<tr>
<td>Group 2</td>
<td>48 (28.8%)</td>
<td>176 (51.8%)</td>
<td>66 (19.4%)</td>
<td>5.052 P=0.030</td>
<td>0.547</td>
<td>4.157 P=0.041</td>
</tr>
<tr>
<td>Group 3</td>
<td>88 (31.2%)</td>
<td>149 (52.8%)</td>
<td>45 (16.0%)</td>
<td>10.55 P=0.005</td>
<td>0.576</td>
<td>8.625 P=0.003</td>
</tr>
<tr>
<td>Group 4</td>
<td>83 (29.0%)</td>
<td>149 (52.1%)</td>
<td>54 (18.9%)</td>
<td>5.300 P=0.071</td>
<td>0.551</td>
<td>4.303 P=0.038</td>
</tr>
<tr>
<td>Group 5</td>
<td>78 (27.2%)</td>
<td>149 (51.9%)</td>
<td>60 (20.9%)</td>
<td>2.700 P=0.259</td>
<td>0.531</td>
<td>2.006 P=0.157</td>
</tr>
<tr>
<td>Group 6</td>
<td>43 (29.7%)</td>
<td>73 (50.3%)</td>
<td>29 (20.0%)</td>
<td>2.865 P=0.239</td>
<td>0.548</td>
<td>2.579 P=0.108</td>
</tr>
</tbody>
</table>

Criteria: group 1, most hypertensive case of each family; group 2, (DBP \(\geq 95\) mm Hg or AHD \(\geq 1\)) and (onset \(< 60\) y); group 3, onset \(< 45\) y; group 4, DBP \(\geq 100\) mm Hg or AHD \(\geq 2\); group 5, (DBP \(\geq 95\) mm Hg or AHD \(\geq 1\)) and (BMI \(< 27\) kg/m\(^2\)); and group 6, (DBP \(\geq 100\) mm Hg or ADH \(\geq 2\)) and (onset \(< 45\) y) and (BMI \(< 27\) kg/m\(^2\)).

Discussion

Several lines of argument designate the aldosterone syn-thase gene as a major candidate gene for predisposition to hypertension. These arguments are drawn from the biochemical role of aldosterone synthase and from the occurrence of major blood pressure disorders due to abnormalities of this gene. We specifically developed genetic tools to investigate the role of the aldosterone synthase gene in human essential hypertension and also used previously described markers having a potential functional effect on the regulation of this gene. A highly polymorphic microsatellite marker was identified at 8 kb in the 3’ region of the CYP11B1 gene, located \(\approx 40\) kb from the CYP11B2 gene. This marker was used to map the CYP11B gene between markers D8S1744 and D8S1836 on the long arm of chromosome 8.

This marker was used in a family study involving a large group (n=167) of well-characterized white families with moderate to severe essential hypertension present in at least 2 sibs. In addition to these criteria, confounding factors such as obesity and diabetes or ethnic heterogeneity were avoided. Statistical analyses of the genotype data obtained in these 292 sib pairs were performed with 2 different statistical methods. Both methods are designed to test for an excess of concordance in the affected sib pairs through the use of identity by descent. Both methods seem to have similar power to detect linkage.25 The results did not show any evidence for linkage to hypertension, in either the whole panel or in family subsets selected for the severity or the early onset of hypertension.

We also conducted a case-control study in 380 French hypertensive patients and 293 normotensive control subjects who were analyzed for the 2 frequent polymorphisms of the CYP11B2 gene.19 We did not observe any association of the intron 2 gene conversion with hypertension, since the allele and genotype frequencies were similar in hypertensives and normotensives. Conversely, the T allele of the –344 polymorphism was significantly more frequent in hypertensives (0.56) than in controls (0.48), suggesting an association of this polymorphism with hypertension. Similar results were obtained by comparing genotype frequencies between the 2 groups. We found a significant association of the T allele with hypertension when controls were compared with the most hypertensive person in each family (\(P=0.023\)). This association was more significant when the normotensives were compared with a subgroup with onset of hypertension before the age of 45 years (\(P=0.005\)). In other hypertensive subgroups the frequency of the T allele was also increased, but the increase was not statistically significant (Table 4). When the analysis was limited to those with a BMI \(< 27\) kg/m\(^2\), the association was not significant, suggesting involvement of the allele in a common mechanism leading to increased BMI and hypertension. The existence of such a common mechanism would be consistent with the known effect of excess mineralocorticoid activity, which leads to salt and water retention and hypervolemic hypertension.26

In an independent study, Benetos et al27 observed a similar frequency of the –344T allele in a series of 216 white hypertensive patients (0.56). However, they reported an association between the –344C allele and higher supine plasma aldosterone levels and lower upright plasma renin levels; an unexpected result, given the increased frequency of the T allele in both hypertensive samples. In the present study we have no information concerning the plasma aldosterone and plasma renin levels of our population. To clarify these apparently conflicting data, we genotyped a sample of 117 normotensive subjects for whom serum aldosterone levels were available. In this sample the T allele was associated with higher plasma aldosterone levels (data not shown). The reasons for the difference between our sample and that of Benetos et al are not evident.

Whether the –344C/T polymorphism represents a functional variant of the CYP11B2 gene modulating the expression of the gene is still a matter of debate. In gel shift assays the –344C allele of the promoter binds the transcription factor SF-1 4 times more than the –344T allele does.19 The regulatory elements of the CYP11B2 gene promoter, required for both basal and angiotensin II– or K+-stimulated transcription, have been mapped in a study with serial deletion mutants.28 A major element is located between –129 and –114 and consists of an SF-1 and chicken ovalbumin upstream promoter-1 binding site. An-
other major regulatory element is located between $-71$ and $-64$ and is necessary for calcium induction of CYP11B2 transcription. In contrast, the $-344$ region, which was confirmed to bind SF-1 in the same study, did not seem to be functionally relevant in their experiments. Hence, binding of SF-1 to this site in vivo is not expected to lead to increased CYP11B2 transcription and may in fact compete with the functional sites for the transcription factor. Under these circumstances the $-344T$ allele, which has reduced affinity for SF-1, would be associated with increased CYP11B2 transcription. The $-344C/T$ polymorphism may be directly implicated in transcriptional control in vivo, or it could be a marker for an as-yet-unidentified polymorphism. In the latter case, the identification of other polymorphisms in the regulatory regions of the gene could be important.

There is an apparent discrepancy between the negative results from the sib-pair study and the positive results in our association study. We also observed discordant results between these 2 approaches in analyses of the angiotensinogen (AGT) and the type 1 angiotensin II receptor (AGTR1) genes. Sib-pair studies are considered to be more robust but to have lesser power than association studies for detecting the implication of a locus in complex diseases. A linkage analysis may be more powerful if genetic heterogeneity is limited by the selection of hypertensive cases based on their plasma levels of aldosterone or K$^+$. However, biological measurements are often difficult to interpret in hypertensives under medication. On the other hand, association studies, while more powerful, may be the source of spurious associations when the 2 samples are not drawn from the same population or when they differ in ethnic origin. Indeed, White and Slutsker previously reported significant ($P<0.001$) racial differences in the frequencies of the 3 different CYP11B1 haplotypes ($344C/intron 2$ without conversion; $344T/intron 2$ conversion; and $344T/intron 2$ without conversion) by comparing 168 white and 73 black schoolchildren. These ethnic differences in allele frequencies of the $344C/T$ polymorphism have also been observed between Africans and whites (X.J., unpublished data). Consequently, although we took into account these possible drawbacks during our enrollment of hypertensive and normotensive individuals, it is important to replicate the positive results of association with the $344T$ allele in independent studies.

It is also possible that the blood pressure effect of the CYP11B2 gene is more prominent in subjects with a high-sodium or low-potassium diet. To test this hypothesis, an independent study population with sufficient information on dietary electrolyte intake would be required.

In conclusion, although we found a significant association of the aldosterone synthase gene with hypertension, our data do not support a major role for this gene in hypertension, at least in whites. In view of the importance of this gene in aldosterone biosynthesis and hence in sodium and water homeostasis, our results need to be compared with other independent studies to define more precisely its implication in the development of hypertension.
17. SAGE: Statistical Analysis for Genetic Epidemiology, release 2.2. SIBPAL version 2.5. New Orleans, La: Department of Biometry and Genetics, LSU Medical Center; 1994.
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