Rationale: The genetic mechanisms underlying hypertension are unclear, but relative aldosterone excess, present in \( \approx 10\% \) of hypertensive patients, is known to be a heritable trait. This phenotype associates with a T/C single nucleotide polymorphism (SNP) at position -344 of the aldosterone synthase gene \((CYP11B2)\). However, deletion of this SNP has no effect on gene transcription. We have identified another T/C SNP at -1651, in tight linkage disequilibrium with the -344 SNP and here investigate its functional effect on \( CYP11B2 \) transcription.

Objective: We assessed the effect on transcriptional activity of the -1651 T/C SNP in vivo and in vitro and propose the mechanism by which transcriptional activity is altered.

Methods and Results: We demonstrated that the SNP at -1651 exerts significant allele-dependent effects on \( CYP11B2 \) transcription. We confirm binding of the transcriptional repressor APEX1 to -1651T, which is associated with reduced transcriptional activity in relation to the less strongly bound -1651C. We show that inhibiting APEX1 by small molecule inhibition or small interfering RNA (SiRNA) leads to increased \( CYP11B2 \) transcription. In addition, overexpression of APEX1 is associated with reduced transcriptional activity. Finally, we also show that -1651T associates with lower excretion rates of aldosterone metabolites in human subjects.

Conclusions: We conclude that APEX1 is a novel transcriptional repressor of \( CYP11B2 \) and that differential APEX1 binding at -1651 of \( CYP11B2 \) results in altered gene expression. This mechanism may contribute to the observed relationship between \( CYP11B2 \) genotype and aldosterone phenotype in a subgroup of hypertensive patients. (Circ Res. 2012;111:00-00.)

Key Words: hypertension \[ \text{aldosterone} \] \[ CYP11B2 \] \[ APEX1 \]

Hypertension is the leading modifiable risk factor for cardiovascular disease, but its etiology remains unclear. Blood pressure is a heritable trait, yet despite extensive genome-wide association studies involving thousands of subjects and examining millions of SNPs, only a small proportion of blood pressure heritability can be accounted for. The relatively few key modifier genes that such studies have managed to identify may be explained, in part, by their lack of detailed subphenotyping. In this regard, approximately 10% of hypertensive subjects have an elevated aldosterone-to-renin ratio (ARR), and it is likely that mechanisms governing the regulation of aldosterone play an important part in the development of hypertension in these subjects. The high heritability of both aldosterone and the ARR are consistent with their genetic regulation. A C/T single nucleotide polymorphism (SNP) in the promoter region of the aldosterone synthase gene \((CYP11B2)\), at position -344 (rs1799998), has previously been associated with hypertension and plasma aldosterone levels in urine and plasma. Although this locus was not identified in GWA studies of hypertension, the size and scale of these investigations preclude detailed subphenotyping, including accurate indices of aldosterone production.

More focused investigations showed association between variation at \( CYP11B2 \), blood pressure, and indices of aldosterone production, while a comprehensive meta-analysis found an association with hypertension and elevated ARR. However, deletion of the -344 C/T site does not
significantly affect promoter activity in vitro. The locus encompassing \( CYP11B2 \) is one of high linkage disequilibrium (LD); therefore, it seems plausible that the true causal variant underlying this hypertensive effect is in LD with the -344 SNP, which is acting as a marker. We demonstrate here that a candidate \( CYP11B2 \) C/T SNP at position -1651 (rs13268025) is in strong LD with the -344 SNP, and that allelic variation at -1651 associates with reduced transcriptional activity in vitro. The -1651 SNP is encompassed by a binding site for the multifunctional protein DNA (apurinic or apyrimidinic) lyase, also called APEX 1 (or, variously, APE1, HAP1, or REF1). Here, we provide evidence for its differential binding to the contrasting -1651 alleles and demonstrate its action as a transcriptional repressor of \( CYP11B2 \). Finally, we show that the -1651 allele associates with altered excretion of urinary aldosterone metabolites in man.

### Methods

#### Reporter Gene Assays

H295R cells were grown in DMEM/F12 medium supplemented with 2% Ultraser G serum (Pall Scientific), 1% ITS, and 1% penicillin/streptomycin. Transfections were performed using siPORT™ NeoFX™ Transfection Agent (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol.

**Reporter Gene Assays**

<table>
<thead>
<tr>
<th>Non-standard Abbreviations and Acronyms</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arr</td>
<td>aldosterone to renin ratio</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>CYP11B2</td>
<td>aldosterone synthase gene</td>
</tr>
<tr>
<td>LD</td>
<td>linkage disequilibrium</td>
</tr>
<tr>
<td>THAldo</td>
<td>tetrahydroaldosterone</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>NRSAT/ SF1</td>
<td>steroidogenic factor 1</td>
</tr>
<tr>
<td>APEX1</td>
<td>apurinic/apyrimidinic endonuclease</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding</td>
</tr>
<tr>
<td>ATF</td>
<td>activating transcription factor AP1 activator protein</td>
</tr>
<tr>
<td>nCARE</td>
<td>negative calcium response elements</td>
</tr>
</tbody>
</table>

Nuclear protein from confluent H295R cells was obtained as detailed in the Online Supplement. Nuclear protein samples contained 1.8kb of \( CYP11B2 \) promoter and were identical apart from the inclusion of a C or T at the -1651 position. ChIP assays were performed using modifications of previous methods.

**Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear protein extraction preparation was modified from Dignam et al., and details are given in the Online Supplement. Nuclear protein was added to a \( ^{32}P \)-labeled double-stranded DNA probe encompassing the -1651 site with binding buffer (see Online Supplement). After 30-minute incubation at room temperature, samples were analyzed by fractionation on a nondenaturing 6% (wt/vol) polyacrylamide gel followed by autoradiography. The EMSA was repeated 3 times, twice with protein aliquots from the same nuclear extraction and a third time with a second, independently extracted protein sample.

**Biotinylated Oligonucleotide Pull-Down Assay**

Nuclear protein from confluent H295R cells was obtained as detailed in the Online Supplement. We incubated 400 μg nuclear protein at room temperature with buffer as described before further incubation with double-stranded biotinylated oligonucleotide either encompassing the -1651 SNP site or containing scrambled sequence. Resulting protein–DNA complexes were captured by incubation with neutravidin-conjugated agarose.

**Tandem Mass Spectroscopy**

SNP-binding proteins were purified, as above. Samples were separated by SDS PAGE (4%–15% gradient gel) and stained with colloidal coomassie blue. Gels were destained and protein bands then excised and sent to the Fingerprints Proteomics Facility, University of Dundee (Dundee, UK). Samples were processed by trypsin digestion and peptides identified from their mass fingerprint using Mascot.

**Western Blotting**

Proteins captured during the biotinylated oligonucleotide pull-down assay were eluted from the beads by a 30-minute, 37°C incubation in 50 μL Laemmli buffer. Samples were then fractionated by SDS-PAGE, transferred, and probed with APEX1 antibody (Abgene, UK), as described in the Online Supplement. We ran 10% of the original extract alongside the pull-down samples as both a positive control and an indicator of relative binding of APEX 1 to our oligos.

**Chromatin Immunoprecipitation Assays (ChIP)**

ChIP assays were performed using modifications of previous methods and are described in full in the Online Supplement. Briefly, chromatin was harvested from confluent H295R cells fixed in 1% formaldehyde. Chromatin was sheared to fragments of ~500 bp genomic DNA. Samples were precleared using protein G sepharose (Sigma, St. Louis, MO), and then subjected to overnight immunoprecipitation at 4°C with APEX1 antibody (Novus Biologicals, Littleton, CO) or nonimmune control serum. Following washing and reversal of cross-links, we analyzed samples by \( CYP11B2 \) promoter-specific RT-PCR, which amplified a 128 bp fragment spanning the -1651 SNP site, using an ABI 7900 HT Prism Sequence Detection System (Applied Biosystems).

**Human Genetic Analysis and Steroid Phenotype**

Ethical approval was granted by the West Glasgow Ethics Committee, and written informed consent was obtained from all participants; investigations were carried out in accordance with the principles of the Declaration of Helsinki. Calculations showed that 60 volunteers would achieve 0.80 power to detect a difference of 20% in urinary tetrahydroaldosterone (THAldo) excretion rate, with \( \alpha \) of 0.05. Sixty volunteers, in good health, ages 18 to 70 years and not on any antihypertensive or steroid-containing medication, were recruited from the local community. Subjects were instructed to adhere to a standard salt diet for 4 days; written
Sixty normal volunteers were recruited for detailed genotyping across the CYP11B2 promoter locus. TSS is transcriptional start site; rs no. is reference SNP number; MAF is minor allele frequency; % genotype is percentage genotyped successfully; Obs Het is observed heterozygosity; HW P value is Hardy–Weinberg P value for SNPs studied, as calculated by Haplovie V4.2.

**Table 1. Genotyping Data**

<table>
<thead>
<tr>
<th>Location (in Relation to TSS)</th>
<th>SNP (rs no)</th>
<th>Allele</th>
<th>MAF</th>
<th>% Genotyped</th>
<th>Obs Het</th>
<th>HW P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1667</td>
<td>rs13254375</td>
<td>G/C</td>
<td>0.47</td>
<td>95</td>
<td>0.56</td>
<td>0.53</td>
</tr>
<tr>
<td>-1651</td>
<td>rs13268025</td>
<td>T/C</td>
<td>0.47</td>
<td>95</td>
<td>0.56</td>
<td>0.53</td>
</tr>
<tr>
<td>-1513</td>
<td>rs62524561</td>
<td>T/C</td>
<td>0.47</td>
<td>95</td>
<td>0.56</td>
<td>0.53</td>
</tr>
<tr>
<td>-1472</td>
<td>rs62524560</td>
<td>A/G</td>
<td>0.48</td>
<td>95</td>
<td>0.58</td>
<td>0.38</td>
</tr>
<tr>
<td>-663</td>
<td>rs28659182</td>
<td>T/A</td>
<td>0.49</td>
<td>98.3</td>
<td>0.54</td>
<td>0.74</td>
</tr>
<tr>
<td>-645</td>
<td>rs11781082</td>
<td>C/T</td>
<td>0.23</td>
<td>98.3</td>
<td>0.39</td>
<td>0.74</td>
</tr>
<tr>
<td>-470</td>
<td>rs11781082</td>
<td>T/C</td>
<td>0.49</td>
<td>100</td>
<td>0.55</td>
<td>0.65</td>
</tr>
<tr>
<td>-344</td>
<td>rs1799998</td>
<td>C/T</td>
<td>0.49</td>
<td>100</td>
<td>0.55</td>
<td>0.65</td>
</tr>
</tbody>
</table>

In order to assess whether the polymorphic variation at this site could alter DNA–protein interactions, an electromobility shift assay (EMSA) was undertaken using H295R nuclear extracts together with -1651 C or -1651 T oligonucleotide probes. Nuclear proteins were prepared using buffers with varying sodium chloride conditions (as this can affect DNA–protein binding). Clear differences in DNA–protein binding were seen in the presence of the T allele or the C allele (Figure 1C) and, while the pattern of oligonucleotide–protein binding changed when the properties of the extraction buffer was altered, particularly with reference to the higher molecular weight band, the complexes of lower molecular weight showed the same allele-dependent difference under all extraction conditions. This confirmed that the presence of the T or C variant had the potential to alter nuclear protein binding at this site.

Rather than use the EMSA to identify proteins differentially bound to each allele by attempting to “supershift” complexes with antibody to a nominated protein, H295R nuclear extracts were incubated with 5'-biotinylated double-stranded DNA probes and streptavidin-agarose beads. The resulting protein–DNA complexes were separated by SDS-PAGE. Two discrete bands were identified in the presence of the T allele and not in the C allele. Following trypsin digestion, the peptide fragments were analyzed by tandem mass spectrometry (FingerPrints Proteomics Facility College of Life Sciences, University of Dundee). Fragments corresponding to the peptide sequence of the transcription factor hnRNPK were identified in one of the protein–DNA complexes. This transcription factor was not investigated further in this work. In the other protein–DNA complex, APEX1 was identified. A biotinylated pull-down assay using an antibody specific to APEX1 confirmed APEX1 binding to oligonucleotides spanning the -1651 SNP; APEX1 did not associate with scrambled control oligonucleotides. This assay indicated that APEX1 is bound to both the -1651C oligonucleotide and genome-wide databases (http://hapmap.ncbi.nlm.nih.gov/, http://www.ensembl.org/index.html), we confirmed the pattern of linkage disequilibrium in the region. While all such SNPs were shown to potentially alter DNA–protein interaction, the polymorphism at -1651 was suggested to influence binding of transcription factors implicated in the regulation of steroidogenesis, including NR5A1 (steroidogenic factor 1, SF-1). Thus, the -1651 SNP was selected for further study.

**Effect of the -1651 SNP on CYP11B2 Transcription and Protein: DNA Binding**

Reporter gene assays were undertaken to assess the effect of polymorphic variation at -1651. Two plasmids were constructed, each containing 1880 bp of the CYP11B2 promoter fused to a luciferase reporter gene. These plasmids were identical except for a single base change at position -1651 (T/C). The plasmids were transfected separately into H295R, a human adrenocortical carcinoma cell line. Under basal conditions, the -1651C plasmid was found to have greater transcriptional activity than the -1651T plasmid (Figure 1A). As expected, incubation of H295R cells with angiotensin II (1×10^{-7} M), the principal troph of aldosterone, stimulated the transcription of both constructs, but the raised level of -1651C transcription over -1651T was even more pronounced (Figure 1B).

In order to determine whether the polymorphic variation at this site could alter DNA–protein interactions, an electromobility shift assay (EMSA) was undertaken using H295R nuclear extracts together with -1651C or -1651T oligonucleotide probes. Nuclear proteins were prepared using buffers with varying sodium chloride conditions (as this can affect DNA–protein binding). Clear differences in DNA–protein binding were seen in the presence of the T allele or the C allele (Figure 1C) and, while the pattern of oligonucleotide–protein binding changed when the properties of the extraction buffer was altered, particularly with reference to the higher molecular weight band, the complexes of lower molecular weight showed the same allele-dependent difference under all extraction conditions. This confirmed that the presence of the T or C variant had the potential to alter nuclear protein binding at this site.

Rather than use the EMSA to identify proteins differentially bound to each allele by attempting to “supershift” complexes with antibody to a nominated protein, H295R nuclear extracts were incubated with 5'-biotinylated double-stranded DNA probes and streptavidin-agarose beads. The resulting protein–DNA complexes were separated by SDS-PAGE. Two discrete bands were identified in the presence of the T allele and not in the C allele. Following trypsin digestion, the peptide fragments were analyzed by tandem mass spectrometry (FingerPrints Proteomics Facility College of Life Sciences, University of Dundee). Fragments corresponding to the peptide sequence of the transcription factor hnRNPK were identified in one of the protein–DNA complexes. This transcription factor was not investigated further in this work. In the other protein–DNA complex, APEX1 was identified. A biotinylated pull-down assay using an antibody specific to APEX1 confirmed APEX1 binding to oligonucleotides spanning the -1651 SNP; APEX1 did not associate with scrambled control oligonucleotides. This assay indicated that APEX1 is bound to both the -1651C oligonucleotide...
Confirmation that APEX1 Is a Transcriptional Repressor of Human CYP11B2

In order to investigate the precise impact of APEX1 on transcription, the effect of E3330, a small molecule inhibitor of APEX1, was assessed using the reporter plasmids described above. Under basal conditions, the addition of E3330 tended to increase the transcriptional activity of both plasmids, but this failed to achieve statistical significance (Figure 2A). However, following stimulation with angiotensin II (1×10^{-7} M), the -1651T plasmid demonstrated increased transcriptional activity in the presence of APEX1 inhibitor, relative to its control, an effect that was not seen with the -1651C plasmid (Figure 2B).

The role of APEX1 as a transcriptional repressor was further confirmed using siRNA knockdown of APEX1, verified by Western blotting (Figure 2C); this resulted in significantly increased luciferase gene expression by both constructs. In addition, overexpression of APEX1 by cotransfection of an APEX1 expression vector (Figure 2D) demonstrated the expected decrease in transcriptional activity of the reporter plasmids.

In summary, these data are consistent with the hypothesis that APEX1 functions as a negative regulator of CYP11B2 transcription. Inhibition of this protein therefore leads to up regulation of CYP11B2 transcription, and overexpression is associated with reduced transcriptional activity. Consistent with the data presented in Figure 1D, where APEX1 is seen to bind to oligonucleotides containing both the C and T allele (albeit with to a greater degree in the T allele), there is a clear effect of manipulating APEX1 in both the -1651T and -1651C reporters constructs.

Functional Effect of the -1651 SNP in vivo

The relationship between aldosterone secretion and the -1651 SNP (in combination with other, linked SNPs in the CYP11B2 5′ regulatory region) was investigated in 60 normal subjects. Demographic data for study participants are shown in Table 2. Individuals adhered to a standardized salt diet, which was designed to provide 100 mmol sodium per 24 hours, for a 3-day period. On the final day, 24-hour urine samples were collected, and subjects with urinary sodium excretion >150 mmol per 24 hours were excluded from further analysis. Urinary sodium excretion of participants homozygous for either the -1651T or -1651C allele was compared, and no significant difference was observed between these groups. Urinary aldosterone metabolites were measured and mean tetrahydroaldosterone (THAldo) for TT subjects 36.10±20.04 μg per 24 hours, TC subjects 48.98±24.30 μg per 24 hours, and CC subjects 57.14±24.00 μg per 24 hours are shown in Figure 3, with THAldo found to be significantly lower in the TT group than the CC group.
and presented as mean±SEM (n =3), analyzed by a Student t test (*P<0.05). Response of reporter gene expression of -1651 T and -1651T plasmid in presence or absence of overexpression of APEX1 following stimulation with 1×10⁻⁷ M angiotensin II (B). Data are shown as firefly luciferase bioluminescence (expressed as relative light units) normalized to a renilla luciferase control, and presented as mean±SEM (n =6), analyzed by a Student t test (*P<0.05). Results are derived from a representative experiment, repeated on 3 independent occasions. For siRNA experiments (C), H295R cells were transfected with -1651T or -1651C plasmids in the presence of control or APEX1-targeting siRNA. Data are shown as firefly luciferase bioluminescence (expressed as relative light units) normalized to protein. Reduction in APEX1 levels was verified by Western blotting (right-hand panels). Results are derived from a representative experiment conducted on 3 independent experiments

(P<0.05). Despite the trend for increased THAldo (TT<TC<TT) - analysis by analysis of variance (ANOVA) was not statistically significant.

Discussion

The CYP11B2 gene is a logical candidate that might plausibly contribute to the phenotype of hypertension and relative aldosterone excess, given that its enzyme product, aldosterone synthase, regulates an important rate-limiting step in aldosterone production. The C/T SNP at -344 of CYP11B2 has been extensively investigated; although there is considerable heterogeneity among different ethnic groups, a meta-analysis indicated that individuals homozygous for -344C have a 17% lower risk of hypertension than do individuals homozygous for -344T. However, deletion of this site showed no significant effect on in vitro gene transcription, and the mechanism underlying its association with hypertension remains obscure. We hypothesized that the -344 SNP acts as a marker for a causal variant elsewhere in the 5’ regulatory region of CYP11B2. We have shown that the -1651 SNP is in strong LD with the -344 SNP in a Caucasian population and present data supporting its functional impact on CYP11B2 transcription through altered binding affinity of the novel transcriptional regulator, APEX1. We have also shown that the -1651 SNP is associated with altered excretion of the principal metabolite of aldosterone (THAldo) in vivo with subjects homozygous for -1651C having higher levels of THAldo than their homozygous -1651T counterparts. Urinary THAldo excretion has the advantage over plasma concentrations of aldosterone in being an integration of aldosterone production over a 24-hour period. As such, it is not subject to the degree of variation that is observed in plasma measurements, even under carefully controlled conditions as in these studies, (posture, sodium intake, time of day, etc). Thus, this provided strong evidence that the polymorphism

Table 2. Demographic Data of 60 Normotensive Volunteers

<table>
<thead>
<tr>
<th>Demographic Characteristics</th>
<th>-1651 TT</th>
<th>-1651 TC</th>
<th>-1651 CC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, † years</td>
<td>45.0±16.1</td>
<td>49.7±18.22</td>
<td>56.5±13.4</td>
<td>NS</td>
</tr>
<tr>
<td>Gender‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>15</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>18</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>SBP, † mm HG</td>
<td>120.4±15.0</td>
<td>128.8±14.33</td>
<td>127.3±12.2</td>
<td>NS</td>
</tr>
<tr>
<td>DBP, † mm Hg</td>
<td>75.0±9.2</td>
<td>74.85±8.4</td>
<td>80.4±7.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as mean±so, with statistical analysis by †analysis of variance or ‡χ². NS, not significant.

Figure 3. In vivo studies of aldosterone production in normal volunteers. Urinary 24-hour THAldo excretion (µg per 24 hours) according to -1651 SNP genotype. TT, CC homozygote individuals. Data are presented as mean±SD, with statistical analysis comparing TT and CC homozygote individuals by Student t test. Comparison of TT, TC, and CC individuals by analysis of variance demonstrates no statistically significant difference.
at -1651 associated with an intermediate phenotype. These in vivo data are consistent with the in vitro findings: -1651C reporter gene constructs have a higher level of expression than do -1651T constructs. Importantly, these data are also internally consistent with earlier studies; -1651C, which binds APEX1 with reduced affinity and is associated with higher aldosterone metabolite excretion, is in very tight LD with the -344T variant previously associated with increased aldosterone levels and greater risk of hypertension.10

In view of the finding that altered reporter gene expression is determined by a single polymorphic variant at -1651, we carried out a series of studies to understand the putative mechanism. Previous investigations have contributed significantly to our understanding of CYP11B2 transcriptional regulation,13,19–21 but few have examined fur-
In summary, we have shown that APEX1 is a novel repressor of CYP11B2 transcription. Furthermore, alterations in CYP11B2 expression that associate with the -1651 SNP may be attributable to differential APEX1 binding due to the disruption of a predicted binding site at this position. In vivo, we demonstrated that the allele predicted to bind APEX1 with greater affinity associates with reduced excretion of aldosterone metabolites, and this is consistent with a transcriptionally repressive role of APEX1. APEX1 is a key enzyme in DNA repair and as such has been the focus of investigations examining the molecular mechanisms of a variety of malignant conditions. Clearly, this would be a major hurdle to the progress of targeting APEX1 in the future as a possible strategy for managing blood pressure. Nevertheless, a greater understanding of the mechanism by which APEX1 regulates CYP11B2 expression would be enlightening and may lead to novel therapeutic approaches.

Acknowledgments
We wish to thank Prof. W.E. Rainey (Department of Physiology, Medical College of Georgia) for the gift of the H295R cells.

Sources of Funding
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Disclosures
None.

References


**Novelty and Significance**

### What Is Known?
- Hypertension is a heritable trait, but despite extensive investigation, many genetic influences on blood pressure are still to be identified.
- The enzyme aldosterone synthase, expressed in the zona glomerulosa of the adrenal cortex, catalyses a rate-limiting stage in the biosynthesis of aldosterone, a key regulator of blood pressure that has highly heritable levels of production.
- A single nucleotide polymorphism (SNP) at position -344 of the aldosterone synthase gene promoter associates with hypertension and elevated indices of aldosterone production, but the mechanism underlying this genotype-phenotype association remains unclear.

### What New Information Does This Article Contribute?
- The -344 SNP in the aldosterone synthase gene is in strong linkage disequilibrium with a C/T SNP at position -1651 of the same gene.
- The -1651 T allele, in comparison to the C allele, associates with reduced excretion of aldosterone metabolites in a cohort of normal volunteers, and with reduced transcriptional activity of the aldosterone synthase promoter in vitro.
- The -1651 T allele has greater affinity for the multifunctional protein APEX1 than does the C allele, and we conclude that this nuclear protein is a novel negative regulator of aldosterone synthase gene transcription.

Despite extensive investigation, many of the mechanisms underpinning the genetic regulation of blood pressure and aldosterone production remain obscure. This is due, in part, to difficulties in characterizing the intermediate phenotype of hypertension with relative aldosterone excess. A SNP at position -344 of the aldosterone synthase gene has previously been associated with hypertension and relative aldosterone excess in studies performed using carefully phenotyped participants. However, this SNP is not functional, and further investigation has sought to understand the mechanism underlying this genotype-phenotype relationship. We have identified a SNP at position -1651 of the same gene, which is in strong linkage disequilibrium with the SNP at -344. This variation at -1651 associates consistently, in an allele-dependent manner, with aldosterone production in vivo, and with altered transcriptional activity of the aldosterone synthase gene in vitro. Our data show that these associations derive from differential binding of the multifunctional protein APEX1 at the polymorphic site, indicating a previously unknown role for this protein as a transcriptional repressor of steroidogenic enzyme expression.

This study demonstrates how a common polymorphic variant can lead to a functional change in gene expression that translates into an important physiological phenotype with significant blood pressure effects.
APEX1 Regulation of Aldosterone Synthase Gene Transcription Is Disrupted by a Common Polymorphism in Humans
Frances McManus, William Sands, Louise Diver, Scott M. MacKenzie, Robert Fraser, Eleanor Davies and John M. Connell

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Supplemental Material

Methods

Reporter gene assays

H295R Cells were grown in DMEM/F12 medium supplemented with 2% Ultroser G serum (Pall scientific), 1% ITS and 1% Penicillin/ streptomycin at 37°C, 5% CO2 until approximately 80% confluent, then transfected with 250ng pGL3-derived expression vectors (Firefly luciferase) (Promega) using 1.5μl siPORT™ NeoFX™ Transfection Agent (Applied Biosystems) according to the manufacturer’s protocol. The pGL3 vector contained sequence corresponding to 1879bp upstream from exon 1 of CYP11B2 (GRCh37:8:143991975:144001138) 2.5ng pGL4.73 (renilla luciferase) (Promega Corp) was co-transfected to control for transfection efficiency. After 24 hours, transfectant was removed and replaced with normal media or, depending on the experiment, with media containing angiotensin II (1x10^-7M) or the APEX1 inhibitor E3330 (10μM final conc.) for 24 hours. For siRNA experiments, cells were transfected with siRNA targeted to APEX1/Ref1 (human ref1, sc-29470, Santa Cruz) or with a scrambled control siRNA (control A, sc-37007 Santa Cruz). For over expression of APEX1, cells were transfected with 500ng APEX1 cDNA expression vector (OriGene, SC119121) or control vector (OriGene, pCMV6-XL5).

For all experiments, cells were lysed with 100μl passive lysis buffer, before adding Dual Glo assay reagents (Promega Corp) according to the manufacturer’s instructions and analysing samples on the Lumat LB9507 instrument (Berthold technologies Ltd).

Electrophoretic Mobility Shift Assay

Confluent 10cm dishes of H295R cells were washed three times with ice-cold PBS, then scraped off and pelleted by centrifugation at 400g, followed by lysis with the addition of 0.4 ml/tube Buffer A (10 mM HEPES, pH 7.9, 10 mM potassium chloride, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.625% (v/v) Nonidet P-40, 0.5 mM PMSF, and 10 μg/ml each of soybean trypsin inhibitor and benzamidine). Samples were then centrifuged at 48,000g for 30s at 4°C, and supernatant removed. The pellet was resuspended in 50 μl of Buffer B (20 mM HEPES, pH 7.9, 100mM – 500 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 10 μg/ml each of soybean trypsin inhibitor and benzamidine). Samples were agitated for 15 min at 4°C, centrifuged at 48,000g for 5 min, and the protein content of the supernatant was determined using a Bradford assay. 5μl samples containing equal amounts of protein from each nuclear extract were then added to a 32P-labeled double-stranded DNA probe (10,000 cpm/sample) containing the polymorphic -1651 binding sequences (sense 5’ GTTGGCCAGGCTAGTCTCGAACTCC 3’, antisense 5’ GGAGTTGAGAATAGCCTGGCCAAC 3’, and sense 5’ GTTGGCCAGGGTCTCGAACTCC 3’, antisense 5’ GGAGTTGAGACCCAGCTGCAAC 3’) to give a final reaction volume of 25 μl containing 10 mM sodium HEPES, pH 7.9, 0.1 mM magnesium chloride, 0.1 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol, 50 mM sodium chloride, and 0.625 μg/ml poly(dIdC). After a 30-min incubation at room temperature, samples were analyzed by fractionation on a nondenaturing 6% (w/v) polyacrylamide gel containing 0.5× Tris-borate/ EDTA buffer (45 mM Tris-borate, 1 mM EDTA) followed by autoradiography.
Biotinylated oligonucleotide pull down assay

H259R cells were washed three times with ice-cold PBS before scraping. Cells were pelleted by centrifugation at 400g and lysed by the addition of 0.4 ml/tube Buffer A (50 mM NaCl, 10 mM HEPES pH 8.0, 500 mM sucrose, 0.5 mM spermidine, 0.15 mM spermine, 0.2% TX-100, 0.5 mM PMSF, 100nM Sodium orthoVanadate and 10 μg/ml each of soybean trypsin inhibitor and benzamidine). The samples were then centrifuged at 48,000g for 30 s at 4°C, and the supernatant was removed. The pellet was then resuspended in 1 ml of Buffer C (50 mM NaCl, 10 mM HEPES pH 8.0, 25% glycerol, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM PMSF, 100nM Sodium orthoVanadate and 10 μg/ml each of soybean trypsin inhibitor and benzamidine). The samples were centrifuged once more at 48,000g for 30 s at 4°C, and the supernatant was removed. To extract proteins bound to DNA, the pellet was then resuspended in 50 μl of Buffer D (400 mM NaCl, 10 mM HEPES pH 8.0, 25% glycerol, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM PMSF, 100nM Sodium orthoVanadate, and 10 μg/ml each of soybean trypsin inhibitor and benzamidine). Samples were agitated for 15 min at 4°C, centrifuged at 48,000g for 5 min, and the protein content of the supernatant was determined using a Bradford assay. For pull down assays, 400 μg of extracted protein was used. Samples were then adjusted to contain a final concentration of the following: 25 mM HEPES pH 8.0, 6 mM MgCl₂, 50 mM NaCl, 3.5 mM spermidine, 1 mM DTT, 380 ng/ml poly (dIdC), 380 ng/ml poly (dGdC), 0.15% (v/v) NP40, 10% (v/v) glycerol, 0.5 mM PMSF, 100nM Sodium orthoVanadate and 10 μg/ml each of soybean trypsin inhibitor and benzamidine. The samples were incubated at room temperature for 20 minutes and incubated for a further 20 minutes with 2 μg of double stranded biotinylated oligonucleotide containing the –1651 SNPs or scrambled control. The duplexes used were –1651 T (sense: 5’ BiotinGGTCAGGAGTTCGAGATTAGCCTGGCCAACACGGT3’, antisense: 5’ ACCGTGTGGGACGGCTAATCTCGAAGTCCTGACC) and –1651C (sense: 5’ BiotinGGTGGCAGGAGTTCGAGATTAGCCTGGCCAACACGGT3’, antisense: 5’ ACCGTGTGGGACGGCTAATCTCGAAGTCCTGACC). Protein-DNA complexes were then captured by incubation with 30 μl of neutravidin conjugated agarose at 4°C with rotation for 2 hrs. Samples were washed 4 times in the above buffer and prepared for SDS PAGE and western blotting.

Western Blots

Proteins captured during the biotinylated oligonucleotide pull-down assay were eluted from the beads by a 30-minute, 37°C incubation in 50μl Laemmli buffer. Samples were then fractionated by SDS-PAGE. After transfer to nitrocellulose, membranes were incubated for 1 h at RT in blocking solution (5% (w/v) BSA in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST)), then for 18 h at 4°C with APEXI antibody (Abgene, UK: 1:2000 in blocking buffer). Membranes were then washed and incubated for 1 h at RT with HRP-conjugated secondary antibody (1:1000 blocking buffer). Following further washes with TBST and Tris-buffered saline, immunoreactive proteins were visualized by enhanced chemiluminescence.

Chromatin immunoprecipitation assays (ChIP)

ChIP assays were performed using modifications of the methods of Edelstein et al (1) and Orlando et al (2). Chromatin was harvested from confluent 6-well cultures of H259R cells. Cells
were fixed for 10 mins by addition of formaldehyde to a final concentration of 1%. The chromatin samples were sheared, using a 6mm probe tip, to fragments that contain approximately 500 bp fragments of genomic DNA, using a Vibracell VCX 130 ultrasonic processor set at 30% output for 12 x 20 sec and 5x 20 sec at 20% output. To reduce non-specific interactions, samples were pre-cleared by incubation with 30 ml of 50% slurry of protein G sepharose (Sigma) for 1 hour at 4°C on a rotating wheel. Pre-cleared samples were then aliquoted into 2 eppendorf tubes and subjected to overnight immunoprecipitation at 4°C with an antibody to APEX1 (1:1000) (Novus Biologicals, NB100-101) or non immune control serum. Following washing and the reversal of crosslinks, samples were analysed by RT-PCR using an ABI 7900 HT Prism Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primers used were forward CCTGAGGTCAGGAGTTCGAG and reverse GCCTCAGCCTCCCAAAGTAG. These amplified a 128 bp fragment of the \textit{CYP11B2} promoter containing the polymorphic –1651 site. The amplicon was detected using Human Universal Probe Library probe 77 (Roche Applied Science).

**Sequencing of the \textit{CYP11B2} promoter region**

DNA was extracted using the Autopure LS, Large Sample Nucleic Acid Purification system (Gentra). The 2kb promoter region was amplified by PCR and sequencing undertaken in three separate reactions. The primers (MWG-Biotech, Ebersberg, Germany) used are shown in Supplemental Table 1. PCR reactions were set up in 96-well plates and performed on a Tetrad PTC-225 Thermo Cycler (MJ Research Waltham, Massachusetts, USA). 5μl DNA (5ng/μl) was added to the following reaction mixture: dNTPs 80μM, Primer Forward 400nM, Primer Reverse 400nM, Expand High fidelity Taq polymerase 0.75 μl, buffer 2.5 μl made to a final reaction volume of 20μl. The PCR conditions were as follows: 94°C for 2 min, 94°C for 15 sec, 61.4°C for 30 sec, 68°C for 4 min. Repeat for 9 cycles. 94°C for 15 sec, 61.4°C for 30 sec, 68°C for 4 min (+ 5 sec per cycle), Repeat for 19 cycles. 72°C for 7 min. The PCR product was cleaned up prior to sequencing using the AMPure system (Agencourt, Beverly, MA, USA.)

PCR products were sequenced using Applied Biosystems Big Dye v3.1 cycle sequencing chemistry (PE Applied Biosystems, Foster City, California, USA), Sequencing reactions were set up in 96 well plates with the following components: PCR product 2 μl, Primer (3.2pmol/μl) 1 μl, Sequencing buffer (5x) 3.5 μl, ABI PRISM Big Dye termination v3.1 ready reaction mix 0.5 μl, Nuclease free water 13 μl. Sequencing primers are shown in Table 1. The sequencing reaction was performed on 96 well PTC 225 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) and the conditions were as follows: 96°C for 45 sec, 50°C for 25 min, 60°C for 4 min, Repeat x 24. Incubate at 94°C for 15 min.

The CleanSEQ purification method (Agencourt, Beverly, MA, USA) was utilised to remove reagents of the sequencing reaction prior to automated sequencing. Automated sequencing was preformed on ABI 3730 DNA analyser (Applied Biosystems, Foster City, CA, USA). Results were analysed using SeqScape v2.2 genotyping software (Applied Biosystems, Foster City, CA, USA) and sequences were aligned to a reference sequence based on published sequences of the \textit{CYP11B2} gene (http://www.ensembl.org/index.html). Allele frequencies, Hardy-Weinberg values, haplotypes, and LD plots were generated using Haploview V4.2 (3).
Low sodium diet (80-100 mmol/ day or around 4g)

Subjects were given the following written instructions which were reinforced by the clinical investigator (FM).
1. Only use a pinch of salt in cooking.
2. Do not add salt at the table or to cooked food
3. Avoid very salty foods such as: tinned meat, bacon, cured meats and ham, sausages, meat pastes or pates, smoked fish or tinned fish in brine or tomato sauce, shellfish, limit hard cheese to 125 g (4 oz) per week, salty savoury biscuits, tinned vegetables in brine or with added salt, tinned and packet soups, Oxo, Marmite, Bovril, stock cubes, gravy powder/ granules, Soya sauce, monosodium glutamate, Salted or flavoured nuts and crisps. There are many alternatives to use instead: all fresh and frozen meat, fresh unsmoked fish, tinned fish in oil or water, milk, eggs, cream, yoghurts, cottage cheese, bread, biscuits, rice, pasta, all animal and vegetable fats and oils, all fruits, all fresh or frozen vegetables, tea, coffee, squash, fizzy drinks, fruit juice, home made soups without salt, unsalted crisps and nuts
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Online Table I: Primer sequences for SNPs in CYP11B2 5’ untranslated region.
Online Figure I: Linkage disequilibrium across the CYP11B2 promoter: linkage disequilibrium plot. Solid red squares denote $D'$ value $>0.95$.
Online Figure II: Comparison of previously reported nCaRE sequences and the sequence spanning the -1651 SNP of \textit{CYP11B2}.

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