Mutations in aldosterone synthase gene of Milan hypertensive rats: phenotypic consequences

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Lloyd-MacGilp, Susan A., Lucia Torielli, Stephanie Bechtel, Grazia Tripodi, Celso E. Gomez-Sanchez, Laura Zagato, Rita Bernhardt, and Christopher J. Kenyon. Mutations in aldosterone synthase gene of Milan hypertensive rats: phenotypic consequences. Am J Physiol Endocrinol Metab 282: E608–E617, 2002. First published November 6, 2001; 10.1152/ajpendo.00043.2001.—Using in vitro and in vivo methods, we have demonstrated increased sensitivity of adrenocortical steroidogenesis to ACTH in Milan hypertensive (MHS) compared with normotensive (MNS) rats and have investigated whether this is caused by mutations of steroidogenic enzymes. Genes encoding aldosterone synthase (CYP11B2) and 11α-hydroxylase (CYP11B1) in MHS and MNS have been cloned and sequenced. Nucleotide 752 mutation caused a Q251R substitution in the amino acid sequence of MHS CYP11B2. The phenotype of MHS CYP11B2 alleles, when expressed in COS-1 cells, differed from that of MNS alleles. The relative activities of the three reactions catalyzed by CYP11B2 (11α-hydroxylation of deoxycorticosterone, 18-hydroxylation of corticosterone, and dehydrogenation of 18-hydroxycorticosterone) were estimated after incubation of transfected cells with [14C]deoxycorticosterone and analysis of radioactivity associated with deoxycorticosterone, corticosterone, 18 hydroxycorticosterone, and aldosterone. Both 11α- and 18-hydroxylase activities were lower (19 and 12%, respectively; P < 0.01 and P < 0.05) in cells transfected with MHS compared with MNS alleles, whereas 18-oxidase activity was 42% higher (P < 0.01). To assess the significance of the CYP11B2 mutation in vivo, DNA from F2 hybrid MHS × MNS rats was genotyped. MHS alleles were associated with lower urine volumes in both sexes, lower ventricle weights in male rats, but no difference in systolic or diastolic blood pressures between the sexes. We conclude that a mutation in CYP11B2 may affect aldosterone secretion in MHS; however, under normal environmental circumstances, we were unable to demonstrate any influence of this mutation on blood pressure.

CYP11B genes; adrenocorticotropic hormone; corticosterone

The Milan hypertensive strain of rat (MHS) is widely used as a genetic model of essential hypertension (1, 7). Recently, mutations in genes encoding adducin, a cytoskeleton protein, have been identified in MHS that are responsible for renal cation transport abnormalities accounting for some, but not all, the blood pressure difference when compared with MNS, the normotensive control strain (2). MHS are also characterized by increased adrenocortical activity, which might contribute to blood pressure control (2, 9, 29, 36). Because adducin is expressed ubiquitously and is implicated in signal transduction processes (37), it is possible that increased steroidogenesis in MHS is an intermediate phenotype caused by greater adrenal responsiveness to stimulation with one or more of the major adrenocorticotropic factors (e.g., ACTH, angiotensin II, or K+). However, we have previously shown that patterns of steroids released into the adrenal vein of MHS and MNS are different (11), observations that are akin to, but not identical with, the steroid hormone profiles of salt-sensitive and salt-resistant Dahl rats (34) and those of Lyon hypertensive and normotensive rats (39). In the case of Dahl rats, differences in steroidogenesis have been attributed to mutations in genes encoding enzymes that catalyze the final steps in the biosynthesis of corticosterone and aldosterone (5, 6, 30). The two genes involved, CYP11B1 (encoding 11β-hydroxylase) and CYP11B2 (encoding aldosterone synthase), are highly homologous and lie in tandem on chromosome 7 (15). CYP11B1 and CYP11B2 are similarly juxtaposed in humans and also appear to be important in the genetic determination of blood pressure and cardiovascular function. Notably, crossover mutations between CYP11B1 and CYP11B2 have been identified as the cause of a rare hypertensive disorder, glucocorticoid-suppressable hyperaldosteronism (27).

In view of previous studies showing associations between blood pressure and the CYP11B1/B2 locus, we investigated whether MHS and MNS differ genetically at this locus too, whether there is an association be-
between CYP11B1/B2 genotype and steroidogenic properties, and whether there are any physiological consequences regarding blood pressure control. Accordingly, we have 1) confirmed phenotypic differences in aldosterone and corticosterone synthesis between MHS and MNS; 2) cloned and sequenced both CYP11B1 and CYP11B2 from MHS and MNS; 3) expressed MHS and MNS genes in COS-1 cells to monitor steroidogenic activity, and 4) evaluated the cosegregation of CYP11B1/B2 alleles with blood pressure and associated phenotypes in an F2 population of MHS × MNS crosses.

MATERIALS AND METHODS

Animals. Tissues for gene sequencing studies and isolated adrenocortical cell studies were obtained from rats held at the Field Laboratories, University of Sheffield, UK. Male rats were maintained in a light- and temperature-controlled environment with free access to food and water. Genetic studies and a further in vivo study of adrenocortical function were carried out with animals from colonies of MHS and MNS rats bred and maintained in Milan. For the genetic study, a large F2 population of 121 males and 130 females was used, as described in previous work (2). Blood pressure, left ventricular weights, and urine volume were analyzed. Urine volume was measured over a 24-h period after rats had been acclimatized to individual metabolic cages with free access to a standard diet (including 0.25% NaCl) and distilled water. Blood pressure was measured as previously described (2). Rats of the F2 population and age-matched rats of parental strains were killed by decapitation after ether anesthesia between 9 and 10 AM. Various tissues, including adrenal glands, were removed and weighed, and corticosterone, as previously described (18). Adrenals from further groups of age-matched male MHS and MNS were taken for Western blotting and RT-PCR.

The relationships in vivo between trophic factors and plasma steroid concentrations were also investigated in groups of nine male MHS and MNS rats (aged 12 wk). Blood samples (0.5 ml) were obtained on two occasions, after cannulation of the right carotid artery and when rats were killed by decapitation 48 h after surgery. Samples were collected and centrifuged in chilled containers, and aliquots of plasma were frozen immediately for later analysis. Renin activity was measured by radioimmunoassay as angiotensin I generated when plasma was incubated at 37°C for 30 min (31).

Plasma ACTH was measured using a kit (ACTH Immulite, Diagnostic Products, Los Angeles, CA). Amplification and sequencing of CYP11B1 and CYP11B2. RNA was extracted from MHS and MNS adrenal glands using the RNeasy Mini Kit (Qiagen, Crawley, Sussex, UK). Reverse transcription of total RNA and subsequent PCR amplification of 11β-hydroxylase and aldosterone synthase open reading frames (ORFs) were carried out using the GeneAmp RNA PCR kit (Perkin-Elmer, Warrington, Cheshire, UK) according to the manufacturer’s instructions. Primers were purchased from Oswell DNA Service (Southampton, UK). Primer pairs for CYP11B1 amplification were TCAGCGATTGCCATATCATTTTG (forward) and GGACAGARGTGCGGGTGAGCT (reverse); primer pairs for CYP11B2 amplification were GATATGGATGACCGGTGGTAAGATGGTAATGT (forward) and GGACACGARTGGGCTTGGACT (reverse). Total RNA (1 μg) was reverse transcribed using oligo (dT) primers. Transcription reactions were carried out as follows: 1 × PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3)], 5 mM MgCl2, 1 mM dNTPs, 2.5 μM oligo primers, 1 U RNA polymerase, and 1 U Taq DNA polymerase. reactions were incubated at room temperature for 10 min, at 42°C for 15 min, at 99° for 5 min, and at 4° for 5 min. Amplification reactions were carried out in a Perkin-Elmer Applied Biosystems thermal cycler in 1 × PCR buffer [10 mM Tris-HCl (pH 8.3) and 50 mM KCl], with 2 mM MgCl2, 0.2 mM dNTPs, 40 pmol of both the forward and reverse primers, and 3 U of Ultma DNA polymerase (Perkin-Elmer) in a total volume of 100 μl. Amplification cycles were as follows: initial denaturation at 94°C for 120 s, then 30 cycles at 94° for 45 s, at 63° for 45 s, at 72° for 120 s, and finally at 72° for 10 min. Amplified products were purified using Microcon YM-100 columns (Millipore, Watford, Herts, UK) and cloned into pPCR-Script SK (+) using the PCR-Script Amp Cloning Kit (Stratagene, Amsterdam, The Netherlands). Plasmid DNA was purified using Wizard Plus MiniPrep DNA Purification System (Promega, Southampton, UK), and the DNA was further purified for automated sequencing by ethanol precipitation. Plasmid template was sequenced using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) with primers that were complementary to both CYP11B1 and CYP11B2. Sequence data were assembled using ABI Prism software. Autoassembler 3.1.2. Comparisons of the sequence data CYP11B1 and CYP11B2 from MHS and MNS were made using GCG software (The Wisconsin Package, version 10) at the UK Human Genome Mapping Project Resource Centre (Cambridge, UK).

Plasmid construction of CYP11B2 ORFs for transient cell expression studies. The CYP11B2 ORF from the MNS pPCR-Script SK (+) plasmid was subcloned into the pSVL SV40 late promoter expression vector (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) by use of NotI and SalI restriction enzymes, blunt ended with the Klenow fragment (Amersham Pharmacia Biotech), and ligated into the Smal site of pSVL to yield plasmid pMNS-AS. An A → G nucleotide substitution, position 752 of the CYP11B2 ORF, was introduced into pMNS-AS using the QuikChange Site-Directed Mutagenesis Kit (Strategene). The nucleotide change was introduced using primers (Operon Technologies, Vh Bio, Newcastle upon Tyne, UK) GACTGCTGTCACACGGCCCGTGTTGAGAAGACATTGTT (forward) and CAAAATGTGTTTCCACACCACGCCTGGTGTTGTCGAGCATC (reverse) to yield plasmid pMNS-AS. The presence of the mutation in pMHS-AS and the integrity of the CYP11B2 ORFs from both pMHS-AS and pMNS-AS clones were verified by automated sequencing, as we have described.
Transfection studies. COS-1 cells were cultured in DMEM (without sodium pyruvate, with 2.5 mM glucose and with pyridoxine HCl; Life Technologies), supplemented with 10% FCS, 2 mM l-glutamate, 1 mM sodium pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (DMEM + FCS). Cells were incubated at 37°C with 5% CO₂. Transfections were carried out by using the DEAE-dextran method (Amersham Pharmacia Biotech) as described by Zuber et al. (42), with some slight modifications. Plates (6 cm) were seeded with 6.5 x 10⁵ cells and grown overnight. The DMEM + FCS medium was aspirated, and the cells were incubated with 2 ml DMEM supplemented with 1 mM HEPES, 2 mM l-glutamate, 100 mM sodium pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (DMEM + HEPES) for 2 h. DEAE-dextran medium (5 μl of 50 mg/ml) was added to reach 800 μl DMEM + HEPES. DNA for transfection was added to 200 μl DMEM + HEPES. Cells were cotransfected with 3 μg of bovine adrenodoxin (ADX) and 6 μg of either pMNS-AS, pMHS-AS, or pSVL. The DMEM + HEPES was aspirated, cells were incubated with the DEAE-dextran and DNA containing DMEM + HEPES for 5 h, and then 2 ml DMEM + FCS containing 360 μg chloroquine (Sigma, Poole, Dorset, UK) were added, and the incubation was continued for a further 2.5 h. The medium was aspirated, and cells were subjected to DMSO shock treatment by incubation for 2 min with Hanks’ balanced salt solution (HBSS) containing NaHCO₃ (Life Technologies) and 10% DMSO (Sigma). After removal of the DMSO solution and two washes with HBSS, cells were incubated overnight with 3 ml of DMEM + FCS. Experiments were repeated three times, with duplicate or triplicate transfections within each experiment.

Aldosterone synthase activity. Transfected cells were incubated with 2 ml of DMEM containing 30 μM 11-deoxy corticosterone (DOC; Sigma) and 6 nCi [¹⁴C]DOC (NEN Life Science Products, Hounslow, UK) for 24 h. Steroid metabolites were extracted and separated with chloroform-methanol-water (300:20:1, vol/vol) by high-performance thin-layer chromatography, as described previously (3). Ultraviolet absorption of unlabeled steroid standards was performed to screen for aldosterone synthase activity. Radioactivity was reported as arbitrary units of intensity after phosphorimager analysis of TLC plates (Bio-Imager; FLA-200, Fuji).

Western blotting of CYP11B1 and CYP11B2. Proteins were extracted from adrenal tissue by a method modified from that of LeHoux et al. (24). Whole adrenals were homogenized in extraction buffer containing 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 5 mM EDTA, 2 μg/ml leupeptin, aprotonin, trypsin inhibitor, antipain, and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). The homogenate was centrifuged at 1,500 g for 10 min to remove cellular debris and then at 11,500 g for 15 min. The mitochondrial pellet was resuspended in a small volume of extraction buffer. Protein concentration was assayed with a kit (Bio-Rad Laboratories, Hemel Hempstead, Herts, UK). Mitochondrial protein (20 μg) was separated by PAGE (22) and blotted onto an enhanced chemiluminescence (ECL) membrane (Amersham Pharmacia Biotech). Western blotting techniques were carried out according to standard protocols by use of monoclonal antibodies raised to specific synthetic peptides of aldosterone synthase and 11β-hydroxylase. Antibodies were generated in CD-1 mice immunized by repeated injections with the synthetic multiple antigenic peptide KVRQNARS1TMDVQVQS-LMAP for aldosterone synthase and with the synthetic peptide KNVYRELAEGRQQSC for 11β-hydroxylase conjugated to chicken serum albumin. Splenies from high titer mice were fused to HL-L Friendly myelome, and monoclonal antibodies were prepared by a standard technique. Clones were screened by ELISA with inner mitochondrial membranes prepared from adrenals of sodium-depleted rats immobilized on 96-well plates. Antibodies produced by incubation of hybridomas in roller bottles were concentrated by ultrafiltration. Immunodetection of the antibody reaction in Milan tissues was determined using the ECL detection system. Quantitative analyses were performed using a phosphorimager (Bio-Imager, FLA-200, Fuji).

Competitive RT-PCR. Primers for the amplification of distinct fragments of CYP11B1 and CYP11B2 genes were those designed by Oaks and Raft (33). A 297-bp fragment of CYP11B2 (position 657–954) was amplified using primers ACCATGGATGTCGACCA (forward) and GAGAGCTGCGAGTCGAGTA (reverse). Primers GAGAGCTGCGAGTGTCGAG (forward) and GCTGGGAATGTTCTCATGG (reverse) amplified a 312-bp fragment from CYP11B1 (position 528–840).

A nonhomologous competitor was constructed from a fragment of the onion allinase gene (a gift of Dr. S. Edwards) with the method of Förster (10). Hybrid primers were designed to amplify a 198-bp fragment from a 1.2-kb fragment of the allinase gene. Transient transfection reactions were as follows: initial incubation at 95°C for 12 min in an Eppendorf Mastercycler gradient thermal cycler. The RT reactions were subjected to PCR amplification reactions as follows: initial denaturation at 95°C for 60 s and then 5 cycles at 95°C for 30 s, at 35°C for 30 s, and at 72°C for 30 s; these were followed by 25 more cycles at 95°C for 30 s, at 55°C for 30 s, at 72°C for 30 s, and finally at 72°C for 2 min. Amplified products were purified using Microcon YM-100 columns and cloned into pPCR-Script SK(+) with the PCR-Script Amp Cloning. Plasmid DNA was purified and sequenced with T7 and T3 primers by automated methods, as described above, to verify the sequence of the competitors. Plasmid template was linearized with HindIII (Promega), and in vitro transcriptions were carried out using standard protocols with T3 polymerase (Promega).

RT-PCR reactions were carried out using the Gene Amp Gold RNA PCR Reagent Kit according to the manufacturer’s instructions. Either 10 ng or 300 ng of total RNA, for the RT-PCR of CYP11B1 and CYP11B2 mRNA, respectively, were reverse transcribed with 1 μl of an appropriate concentration of competitor, diluted in 0.05 mg/ml tRNA (Life Technologies, Paisley, UK). Transcription reactions were carried out in 1X RT-PCR buffer (30 mM Tris-HCl and 20 mM KCl, pH 8.3), 2.5 mM MgCl₂, 250 μM dNTPs, 10 mM dithiothreitol, 1.25 μM random hexamer, 10 U of RNase inhibitor, and 15 U of Multiscribe reverse transcriptase in a total volume of 20 μl. Reactions were incubated at 25°C for 10 min and at 42°C for 12 min in an Eppendorf Mastercycler gradient thermal cycle. The RT reactions were subjected to PCR amplification as follows: 1× RT-PCR buffer (30 mM Tris-HCl and 20 mM KCl, pH 8.3), 2 mM MgCl₂, 200 μM dNTPs, 20 pmol primers, and 2.6 U AmpliTaq Gold DNA polymerase. Amplification reactions were as follows: initial incubation at 95°C for 10 min, and then 28 cycles at 48°C for 45 s, 72°C for 45 s, and finally 72°C for 5 min. Amplified products were...
separated on 2% agarose gels. Quantitative analyses were performed using a phosphorimager.

Genotype determination for CYP11B2 and other polymorphic markers. DNA for genotyping F2 rat populations was extracted from tails. PCR and gel electrophoresis for markers on chromosome 7 (D7Rat32, D7Rat44, D7Rat 66, D7Wox3, D7Wox6, D7Mit4, D7Mit5, D7Mit14, D7Mgh1, Cyp11B1) were carried out as previously described (41). CYP11B2 genotyping was based on a single base A-to-G transition in codon 251, which gives rise to a restriction fragment length polymorphism (RFLP) with the restriction enzyme Smal (restriction site absent in MNS and present in MHS). PCR amplification was carried out with primers (forward: 5'GAGATCCACGGCATCAAAAT-3' and reverse: 5'GACATCAGCGCACTTTG-3') based on the exon 4 genomic sequence (GenBank accession no. D14093). The reaction was performed in 20-μl reaction volume under the following conditions: 250 ng genomic DNA, 0.4 μM each primer, 200 μM dNTPs, NH4 buffer, 1.5 mM MgCl2, and 2 U Taq polymerase (Bioline, London, UK). PCR was carried out for 32 cycles with an annealing temperature of 55°C. The genotypes in the F2 (MHS × MNS) intercross cohort were then analyzed by 4% agarose gel electrophoresis after digestion of the PCR amplification products with the restriction enzyme Smal, which results in 159- and 35-bp fragments in the case of the MHS allele; the PCR product from MNS DNA remains uncleaved (194 bp).

Linkage and statistical analysis. Linkage map and quantitative trait loci (QTL) localizations were done with MAPMAKER/EXP and MAPMAKER/QTL 1.1 programs as previously described (41). According to Lander and Kruglyak (23), threshold logarithm of odds (LOD) score values of 1.9 and 3.3 may be considered as suggestive and significant evidence of linkage in a codominant genetic model. One-way ANOVA was used to compare tissue weights of MHS and MNS and steroid output in transfected cells. Plasma concentrations of aldosterone, corticosterone, ACTH, and plasma renin activity in cannulated rats and dose-response curves to ACTH in MHS and MNS ZG and ZF cells were compared by MANOVA. Data from cosegregation studies were analyzed by one-way ANOVA with the Newman-Keuls correction test for multiple comparisons. P values of <0.05 were considered significant. Statistical packages used were Minitab and SPSS.

RESULTS

Tissue weights and adrenocortical function in Milan strain. In rats used for the preparation of adrenocortical cells, MHS had heavier adrenal glands, higher plasma aldosterone concentrations, smaller thymuses, and smaller kidneys compared with MNS (Table 1). Responses of ZG and ZF cells from MHS and MNS to ACTH are shown in Fig. 1. Overall aldosterone responses, when account was taken of fixed and random variables, were greater in MHS compared with MNS ZG cells (P = 0.002), but differences at each concentration of ACTH were not statistically significant. Corticosterone responses of MHS ZF cells were approximately twofold greater than those of MNS cells (P < 0.001). Despite increased aldosterone production by stimulated ZG cells, Western blotting (Fig. 2) indicated that aldosterone synthase tended to be lower in MHS compared with MNS adrenals. Although this differ-

Table 1. Tissue weights and plasma hormone concentrations in male Milan rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>MHS</th>
<th>MNS</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney Wt, g/100 g body wt</td>
<td>0.76 ± 0.01</td>
<td>0.57 ± 0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adrenal Wt, mg/100 g body wt</td>
<td>7.87 ± 0.37</td>
<td>9.08 ± 0.30</td>
<td>0.05</td>
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<tr>
<td>Aldosterone, pM</td>
<td>634.4 ± 123.7</td>
<td>1323.0 ± 219.1</td>
<td>0.001</td>
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<tr>
<td>Corticosterone, nM</td>
<td>33.2 ± 7.1</td>
<td>51.4 ± 10.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Thymus Wt, mg/100 g body wt</td>
<td>1.76 ± 0.09</td>
<td>1.37 ± 0.04</td>
<td>0.002</td>
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Values are means ± SE; n = 8–12. MNS, Milan hypertensive rats; MHS, normotensive rats; NS, not significant.

Fig. 1. Aldosterone (A) and corticosterone (B) responses to ACTH of zona glomerulosa and zona fasciculata cells from adrenal glands of Milan hypertensive (MHS) and normotensive (MNS) rats. Results are from 3 separate experiments, when cells were obtained from glands of 2 rats of each strain. Within each experiment, average incremental hourly steroid production rates from quadruplicate incubations were calculated by subtracting baseline values of cells incubated without ACTH. For zona glomerulosa cells, baseline aldosterone values were 4.0 ± 2.2 and 3.8 ± 1.6 ng/10⁶ cells for MHS and MNS, respectively; for zona fasciculata cells, corresponding baseline corticosterone values were 3.1 ± 1.9 and 2.5 ± 1.4. Values shown are means ± SE of 3 experiments. ANOVA indicated a significantly greater output of aldosterone by MHS cells (P < 0.002). Corticosterone output by MHS cells was significantly greater than by MNS cells (P < 0.001).
ence was not statistically significant ($P < 0.1$), it nevertheless contrasts with the ability of MHS adrenal glands to secrete greater amounts of aldosterone. 11β-Hydroxylase expression was higher ($P < 0.05$) in MHS, which is consistent with data from several sources showing increased corticosterone synthesis compared with MNS (2, 9, 29, 36).

In a further experiment, corticosterone, aldosterone, and ACTH concentrations and plasma renin activities in plasma from rats sampled on three separate occasions (24, 36, and 48 h after cannulation) were compared in MHS and MNS rats. With account taken of repeated measurements and differences due to time of sampling, concentrations of aldosterone and corticosterone overall were higher in MHS than MNS (MANOVA; $P < 0.001$ in both cases); plasma ACTH concentration and renin activities did not differ significantly between strains. When analyzed separately by one-way ANOVA, aldosterone was significantly higher in MHS at each time point ($P < 0.05$, 0.05, and 0.01) but only at the final time point for corticosterone ($P = 0.005$). This time-dependent difference reflects the stress of surgery; plasma ACTH concentrations were reduced by $>60\%$ from the 24-h to the 48-h time point ($579 \pm 105$ to $221 \pm 33$ pg/ml; $P < 0.001$). The relationships between aldosterone and corticosterone concentrations and ACTH and plasma renin shown in Fig. 3 suggest that steroid concentrations are elevated in MHS irrespective of differences in tropic stimulation; plasma aldosterone and corticosterone were correlated with plasma ACTH concentration in MNS ($r^2 = 0.4$ and 0.52, respectively; $P < 0.001$ for both steroids). Correlations between ACTH and steroid concentrations were not statistically significant in plasma samples from MHS.

Figure 4 shows representative RT-PCR results of CYP11B1 and CYP11B2 mRNA expression. Differences between MHS and MNS tissues were not significant for either gene.

**Sequencing.** MHS and MNS nucleotide and amino acid sequences for aldosterone synthase and 11β-hydroxylase were aligned and compared with those published for Sprague-Dawley rats (30, 32). MNS sequences were identical with those of Sprague-Dawley rats, as was MHS CYP11B1. Three nucleotide substi-
tutions were observed in CYP11B2 of MHS. Two of the substitutions in the MHS gene were silent (exon 1, nucleotide 60 G→A; exon 6, nucleotide 1084 C→T). The third substitution (exon 4, nucleotide 752 A→G) caused an amino acid change at position 251 from glutamine→arginine. This gave a restriction site for SmaI in MHS that was used to distinguish MHS and MNS CYP11B2 alleles when F2 rats were genotyped (see Cosegregation studies).

Expression studies in transfected cells. When transfected into COS 1 cells, CYP11B2 alleles of MHS and MNS genotype converted DOC to corticosterone, 18OH corticosterone, and aldosterone. Radioactivity was quantified by phosphorimaging analysis and reported as arbitrary units of intensity. Conversions to corticosterone (MNS: 597.5 ± 63.9; MHS: 583.8 ± 61.5) and aldosterone (MNS: 459.1 ± 90.6; MHS: 439.6 ± 60.6) were similar for MNS and MHS alleles, but 18OH corticosterone release (MNS: 423.1 ± 42.2; MHS: 294.8 ± 14.4) appeared less for MHS alleles (P < 0.02). Bearing in mind that aldosterone synthase catalyzes three sequential reactions (hydroxylations at the 11 and 18 positions and then dehydrogenation of 18OH corticosterone), we assessed individual reactions as ratios of products to substrate. Figure 5 shows that cells transfected with MHS, compared with MNS alleles, had less 11β-hydroxylase activity (P < 0.01; aldosterone + 18OH corticosterone + corticosterone to DOC) and 18-hydroxylase activity (P < 0.05; aldosterone + 18OH corticosterone to corticosterone) and greater 18-dehydrogenase activity (P < 0.01; aldosterone to 18OH corticosterone).

Cosegregation studies. DNA from F2 rats was genotyped according to the SmaI restriction site in MHS CYP11B2 alleles. No significant influence of MHS ge-

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**Fig. 4.** Competitive PCR analyses of CYP11B1 and CYP11B2 mRNA in adrenal glands of MHS and MNS rats. A: representative gels of templates and competitors after coamplification by PCR. Template concentrations were 10 and 300 ng for CYP11B1 and CYP11B2, respectively. B: representative plots used to calculate mRNA levels. Intensities of template and competitor bands were measured using a phosphorimeter. Linear regression was used to plot the relationships between log (template/competitor) and competitor concentration. Points of molar equivalence were calculated (arrows) corresponding to template concentration. C: comparisons of adrenal mRNA levels for CYP11B1 and CYP11B2 expression (n = 6–8) in MHS and MNS tissues expressed per µg of total RNA.

**Fig. 5.** Aldosterone synthase activity of COS-1 cells transfected with MHS and MNS CYP11B2 alleles. Values are means ± SE of 8 separate transfections and are normalized with respect to activity of MNS alleles. Ratios of corticosterone to 11-deoxycorticosterone, 18-hydroxycorticosterone to corticosterone, and aldosterone to 18-hydroxycorticosterone were used to represent 11-hydroxylase, 18-hydroxylase, and 18-oxidase activities.
notype on systolic or diastolic blood pressures was seen, but rats homozygous for the MHS genotype had lower left ventricle weights and urine volumes than MNS homozygotes (Fig. 6). These differences contrast with values in age-matched parental strains, where daily urine volume appeared higher for male MHS than MNS (4.04 ± 0.38 vs. 2.56 ± 0.18 ml/kg body wt; \( P < 0.05 \)), as did left ventricular weights (0.195 ± 0.002 vs. 0.176 ± 0.003 g/kg body wt; \( P < 0.05 \)), particularly in male rats.

DNA samples from F2 rats were screened with 12 markers of chromosome 7, which were polymorphic between MHS and MNS. These data were used to search for QTL for various phenotypes in both males and females and to assess any contribution that the \( \text{CYP11B1}/\text{B2} \) locus might make (Fig. 7). A QTL for urine volume was identified close to D7Mit4, where the LOD score for all rats (4.0) exceeded the significance threshold. When analyzed by gender, only male rats (LOD score: 3.6) showed linkage at this locus. The \( \text{CYP11B1}/\text{B2} \) locus, which is separated from D7Mit4 by \(-25\) cM, had a LOD score of 2.1 for urine volume, which is suggestive of a linkage. However, LOD score values for urine volume at \( \text{CYP11B1}/\text{B2} \) were below this threshold when male and female data were analyzed separately. LOD scores for blood pressure and ventricular weight of chromosome 7, including the \( \text{CYP11B2} \) locus, were less than the threshold indicating the possibility of linkage (i.e., \(<1.9\)).

**DISCUSSION**

We have investigated the basis for increased steroidogenic activity in MHS. At the outset, the phenotype of heavier adrenal glands and higher plasma concentrations of aldosterone and corticosterone, which others have described, was confirmed. A number of possible explanations are suggested: 1) that feedback control of the hypothalamo-pituitary-adrenal axis is impaired; 2) that adrenocortical cells are intrinsically more sensitive to stimulation; 3) that steroidogenic enzymes are more active. The first suggestion is supported by our previous study of glucocorticoid receptor-binding properties, which demonstrated that hepatic GR in MHS exhibit reduced affinity for corticosterone compared with MNS GR (19). If this property were common to all tissues, then diminished negative feedback of ACTH secretion would lead to adrenal cell hypertrophy and hyperplasia, particularly of ZF cells (the predominant adrenal cell type). However, despite impaired GR binding, we have unpublished evidence that hepatic phosphoenolpyruvate carboxykinase, a glucocorticoid-regulated enzyme, is more, not less, active in MHS. It is also significant that thymus weight is less in MHS, another feature of excess glucocorticoid activity.

**Fig. 6.** Blood pressure, urine volume, and left ventricle weight phenotypes in male and female F2 (MNS × MHS) rats according to \( \text{CYP11B2} \) genotype. Values are means ± SE.

**Fig. 7.** Logarithm of odds (LOD) score curves for quantitative trait loci in male and female rats on chromosome 7. LOD thresholds for suggestive (1.9) and significant (3.3) linkage are indicated by solid and dotted horizontal lines, respectively. SBP, systolic blood pressure; LVM, left ventricular mass; UV, urine volume.

**CYP11B1/B2** locus, were less than the threshold indicating the possibility of linkage (i.e., \(<1.9\)).
We have assessed responsiveness of isolated ZG and ZF cells. Both cell types from MHS were significantly more sensitive to ACTH than those of MNS. Similarly, in vivo, plasma aldosterone and corticosterone concentrations were higher in MHS than in MNS over a range of concentrations of plasma ACTH. It is generally acknowledged that acute increases in steroidogenesis are mediated by the supply of substrate, cholesterol, to the mitochondrial, cholesterol side-chain cleavage enzyme. It is relevant, therefore, that more cholesterol is stored in MHS than in MNS adrenal cells (29). However, whether this is a cause or a consequence of raised steroid hormone concentrations is unclear.

Another factor to consider is that hypertension in MHS is primarily due to mutations of adducin, which alter renal cation transport. The mechanism of ACTH action also involves changes in cation transport. Therefore, if we assume that adducin is ubiquitously expressed, it is easy to see that adducin mutations might interfere with signal transduction processes.

**CYP11B1** and **CYP11B2** are key enzymes regulating corticosterone and aldosterone synthesis, respectively. Western blotting and competitive RT-PCR techniques were developed to compare expression in MHS and MNS adrenal glands. Differences in **CYP11B1** and **CYP11B2** mRNA expression between MHS and MNS did not achieve statistical significance, but protein levels were differentially affected. Expression of 11β-hydroxylase was higher in MHS than in MNS, which could account for the greater output of corticosterone from ZF cells stimulated with ACTH. In contrast, aldosterone synthase levels appeared lower; although the difference was not statistically significant. The discrepancy between mRNA and protein expression is difficult to explain, but not without precedent. Rats treated chronically with ACTH show reduced adrenal expression of mRNA of several steroidogenic enzymes without corresponding changes in protein levels (25). It is interesting to note that Hornsby and Crivello (13, 14) suggest that adrenal **CYP450** enzymes are susceptible to oxidative damage and that aldosterone synthase, because of blood supply to the ZG, is particularly exposed. A difference in adrenal blood supply could account for an apparently shorter half-life of aldosterone synthase in MHS adrenals, but not also an increase in 11β-hydroxylase stability, particularly as 11β-hydroxylase in the ZF is relatively protected from peroxidation.

Our previous work with adrenal vein samples found differences in steroid hormone profiles between MHS and MNS that were indicative of altered **CYP11B1/B2** properties. However, measurements of plasma ratios cannot distinguish the zonal origin of adrenocortical steroids, or whether **CYP11B1** and/or **CYP11B2** properties are affected. We chose to compare the coding sequences of each enzyme for MHS and MNS, because this approach had successfully identified several mutations in both genes in the Dahl rat. It seemed less likely that promoter sequences would be affected, given that mRNA expression levels in Milan rats were similar for both genes. The only meaningful difference found was a G-to-A substitution at nucleotide 752 in exon 4 of **CYP11B2**, which caused a glutamine-to-arginine change at amino acid 251. Interestingly, this same mutation was one of two found in Dahl salt-resistant rats (6). In vitro expression studies showed that the MHS gene had little effect on net aldosterone production in COS-1 cells but did appear to reduce 18OH corticosterone output. We acknowledge that this experiment may be flawed because only one relatively high concentration of DOC was tested and because aldosterone production may have been limited by the availability of cofactors in COS-1 cells compared with steroidogenic cell lines. However, our interpretation of these data is that there are reciprocal changes in the properties of aldosterone synthase such that 11/18-hydroxylase activities are decreased, whereas 18-oxidase activities are increased. In contrast, the combined effect of the two Dahl salt-resistant mutations caused a thousandfold increase in aldosterone production by transfected steroidogenic MA-10 cells. Whether this reflects the added influence of the second mutation or differences in protocol for testing enzyme activity is unclear. In vivo, the difference in aldosterone production between resistant and sensitive strains is much more modest.

An attempt has been made to analyze the separate effects of the Dahl resistant type of mutations at corresponding sites in human **CYP11B2** (8). As in our experiments, the production of corticosterone, 18OH corticosterone, and aldosterone from radioactive DOC was analyzed by TLC. Both Dahl-type mutations increased aldosterone synthesis, but again residue 251 appeared to critically affect relative 18-hydroxylase and 18-oxidase activities. Changing lysine (corresponding to glutamine in rat) to arginine at position 251 increased 18OH corticosterone output 4- to 5-fold but increased aldosterone synthesis only 1.5-fold. In vivo, these reciprocal changes would become important only if **CYP11B1** and **CYP11B2** were coexpressed when 11 hydroxylated products from **CYP11B1** could be utilized by aldosterone synthase.

The physiological consequences of the MHS **CYP11B2** mutation were tested with DNA archived from an F2 population of an MHS × MNS cross. Unfortunately, it was not possible to match phenotypic information about steroid hormone levels or adrenocortical activity with **CYP11B2** genotype. However, bearing in mind that MHS exhibit characteristics of mineralocorticoid excess (11), our expectation was that the **CYP11B2** mutation might influence blood pressure, possibly with consequential increases in heart mass. Indeed, given recent findings that aldosterone regulates cardiac function in rats (35) and that a polymorphism in the promoter region of **CYP11B2** is associated with ventricular hypertrophy in humans (21), we considered the possibility that **CYP11B2** might affect heart weight independent of blood pressure in MHS. In fact, blood pressures of F2 rats were similar for all genotypes, and left ventricle weight tended to be lower, not higher, in rats with MHS **CYP11B2** alleles. Given that mineralocorticoid-induced hypertension in ro-
students requires dietary sodium loading and a reduction in renal mass (20), the lack of influence of CYP11B2 on blood pressure might be explained, but the reason for reduced heart weight with MHS alleles is less clear. In some respects, this inconsistency is similar to that of Dahl salt-sensitive and salt-resistant rats. MHS have one of the two mutations in CYP11B2 found in Dahl resistant rats. Paradoxically, the combined CYP11B2 mutations of Dahl-resistant rats cause increased aldosterone synthase activity in vitro, yet they are associated with lower blood pressures and left ventricular weights in vivo. It has been argued, however, that mutations of CYP11B1 rather than CYP11B2 genes contribute to hypertension and left ventricular hypertrophy of Dahl-sensitive compared with resistant rats (4). This cannot account for differences between MHS and MNS, since the sequences of CYP11B1 are identical, although it is possible that other genes in close linkage disequilibrium may be involved. In two separate studies, QTL for left ventricular mass have been identified on chromosome 7 in the vicinity of the CYP11B1/B2 locus (12, 38).

The association of MHS genotype with reduced urine volume was found in males and females. This observation is consistent with known effects of aldosterone (20). Interval mapping on chromosome 7 established a QTL for urine volume near the CYP11B1/B2 locus in male but not in female rats. The LOD scores for CYP11B2 did not meet strict statistical tests of significance but were at least as good as those suggesting linkage of CYP11B1 with blood pressure in Dahl rats and left ventricular mass (4, 5). The possibility that another gene in close linkage disequilibrium is involved cannot be excluded. However, as yet there is no information about alternative candidate genes that could account for LOD scores > 3.5 in the vicinity of D7Mit4.

In summary, we have provided evidence of increased adrenocortical sensitivity to ACTH in MHS compared with MNS rats. A difference in the sequence of CYP11B2 was identified in MHS that altered the relative 18-hydroxylase and 18-oxidase properties of aldosterone synthase and an increase in the expression of 11β-hydroxylase has been found. These differences could account for previously observed steroid profiles in adrenal vein samples of MHS and MNS. The CYP11B2 mutation might influence plasma aldosterone concentration and hence determine heart weight and urine volume. Potentially, elevated plasma aldosterone could influence blood pressure via a mineralocorticoid-dependent mechanism, but the present linkage studies have not identified the MHS CYP11B2 mutation as a determinant of blood pressure. It should be noted, however, that mineralocorticoid-induced hypertension in rats is very much affected by two variables, salt sensitivity and renal mass (16), phenotypes that differ markedly between MHS and MNS. Therefore, a more complete test of the MHS CYP11B2 mutation would require rats to be fed a high-salt diet and should also take account of genetically determined variations in renal mass.

No difference in the sequence of CYP11B1 was identified to account for increased corticosterone synthesis in MHS, but higher levels of hormone and 11β-hydroxylase protein were consistent with greater sensitivity to ACTH. However, in vivo and in vitro studies indicated that acutely both aldosterone and corticosterone responses to ACTH were greater in MHS adrenocortical tissue. This could reflect an enhancement of signal transduction processes, which in turn might contribute to the higher blood pressure of MHS. The work of Whitworth et al. (40) has investigated in some detail the pressor effects of ACTH among five different strains of rat. Blood pressure increases were similar, but responses relating to fluid and electrolyte balance were different (40). Interestingly, although ACTH-induced hypertension is associated with corticosterone synthesis (28), it is not clear that pressor responses are mediated by either mineralocorticoid or glucocorticoid receptors (26).

We conclude that increased adrenocortical activity in MHS is associated with greater sensitivity to ACTH and a mutation in the coding region of CYP11B2. Further tests, which take account of known genetic and phenotypic differences between MNS and MHS, are required to define any cardiovascular consequences of abnormally high steroid hormone concentrations.

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