PHYSIOLOGIC EFFECTS OF CYTOCHROME P450 3A ACTIVITY

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Nutrition, School of Public Health.

Chapel Hill
2007

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The cytochrome P450 3A (CYP3A) subfamily of enzymes comprises isoforms that catalyze metabolism of foreign or "xenobiotic" chemicals that include most marketed pharmaceuticals. CYP3A activities are also critical in clearing and altering the bioactivities of many endogenous molecules. Research into CYP3As has focused primarily on their roles in drug metabolizing; much less attention has gone to physiologic effects of CYP3A activities. I review here established and hypothesized physiologic roles for CYP3A enzymes.

I have specifically studied whether CYP3As modulate renal sodium transport and blood pressure. A single-nucleotide polymorphism (SNP; A6986G) in the CYP3A5 gene determines CYP3A5 apoprotein expression and enzyme activity. A6986G was shown to predict renal CYP3A expression and activity among human kidney samples mostly from Caucasians and to associate with blood pressure and renal function among African-Americans.

Genotyping for A6986G and additional expression-determining CYP3A5 SNPs (G14690A and 27131-32insT) was performed for a larger group of African-Americans. I show that genotypes at these loci can be computationally resolved into
functional haplotypes, which may improve the ability of \textit{CYP3A5} SNPs to predict blood pressure and other phenotypes.

I used mouse kidney collecting duct cells to study effects of a product of \textit{CYP3A} catalysis, 6\(\beta\)-hydroxycorticosterone. I show that 6\(\beta\)-hydroxycorticosterone inhibits activity and expression of 11\(\beta\)-hydroxysteroid dehydrogenase type 2, an enzyme that prevents glucocorticoids from inappropriately activating the mineralocorticoid receptor. 6\(\beta\)-hydroxycorticosterone also appears to antagonize mineralocorticoid receptor-mediated increases in expression of genes related to sodium transport.

Wild-type mice that were injected with 6\(\beta\)-hydroxycorticosterone failed to demonstrate greater sodium-sensitivity of blood pressure than did mice injected with vehicle. Mouse models were made to express human \textit{CYP3A5}. Mice bearing a \textit{CYP3A5} transgene exhibited slightly higher blood pressures than did wild-type mice, an effect that may be sodium-dependent.

Finally, I performed a systematic review and meta-analysis of subsequent human studies of \textit{CYP3A5} genotype associations with blood pressure and hypertension. Considerable variability is apparent among these studies. Intriguing data emerge but summary statistics for suitable studies (primarily of healthy whites) show little evidence for significant association. I discuss the need for increased standardization among future studies and for greater inclusion of black subjects.
DEDICATION

For My Grandmothers

The fathers may soar
And the children may know their names

Toni Morrison, Song of Solomon

*dixit et ignotas animum dimittit in artes naturamque novat*

Ovid, Metamorphoses, VIII
ACKNOWLEDGEMENTS

Nobuyo Maeda is a peerless mentor and a tireless advocate of learning. Her insight, energy, enthusiasm, and support have been critical in the effort to produce this dissertation. Oliver Smithies as well is a fountain of wisdom and example of the need for dogged pursuit in the search for answers to research questions. Their love of science and their work ethic have left an indelible imprint upon me.

Paul Watkins has been an outstanding mentor and dissertation committee member who started me on the path to this project. Committee members John Anderson, Melinda Beck, and Boyd Switzer provided much critical insight and counsel in the progress of this research. I am also indebted to the personal and intellectual legacies of previous mentors Salvatore Pizzo (Duke University) and J. Michael Pierce (University of Georgia).

Members of the Smithies-Maeda laboratory were invaluable in this effort: John Hagaman, Sylvia Hiller, Jenny Langenbach, John Cowhig, Jennifer Wilder, Lonquan Xu, Sharome Jordan, Xianwen Yi, Feng Li, Natalia Makhanova, Nobuyuki Takahashi, Masao Kakoki, Seigo Hatada, Tomoko Hatada, Hyung-Suk Kim, Shinja Kim, Michael Altenburg, Jose Arbones-Mainar, Ray Fox, Lance Johnson, Hind Muallem, Heather Doherty, Avani Pendse, Yau-Sheng Tsai, and Chih-Hong Wang.

I thank the American Physiological Society for granting permission to reprint material in Chapter II. I thank the Doris Duke Charitable Foundation and the National Institutes of Health for crucial financial support. I thank Bruce Stanton (Dartmouth)
for the mIMCD-K2 cell line. I thank Erin Schuetz (St. Jude’s) for CYP3A5 cDNA, for collaboration, and for providing space in her laboratory for me to learn the CYP3A5 genotyping protocol. I thank the UNC Department of Nutrition, and especially Joanne Lee, for invaluable aid and support.

I thank my friends and family for sustaining me to this point. My journey began with a family that valued and promoted education. My mother, Deborah Givens, was a relentless advocate and advisor even if she may not always have understood where my path was taking me. I thank my sister Lauren Givens for her love and support. My partner throughout this process, Monica Copeland, was always available to listen, console, and motivate. The Rhodes family have stood behind and encouraged me throughout my studies. Lastly, I thank St. Paul AME Church (Chapel Hill, NC) and its members for the promise of a new, better, and more purposeful life.
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<th>Definition</th>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AGT</td>
<td>angiotensinogen</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AT-II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>B</td>
<td>corticosterone</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>CAR</td>
<td>constitutive androstane receptor</td>
</tr>
<tr>
<td>CBX</td>
<td>carbenoxolone</td>
</tr>
<tr>
<td>CrCl</td>
<td>creatinine clearance</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>DME</td>
<td>drug-metabolizing enzyme</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>EM</td>
<td>expectation-maximization algorithm</td>
</tr>
<tr>
<td>ENaC</td>
<td>epithelial sodium channel</td>
</tr>
<tr>
<td>F</td>
<td>cortisol</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FXR</td>
<td>farnesyl X receptor</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>HSD11B1</td>
<td>11β-hydroxysteroid dehydrogenase type 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HSD11B2</td>
<td>11β-hydroxysteroid dehydrogenase type 2</td>
</tr>
<tr>
<td>HTN</td>
<td>hypertension</td>
</tr>
<tr>
<td>JGA</td>
<td>juxtaglomerular apparatus</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand-binding domain</td>
</tr>
<tr>
<td>LD</td>
<td>linkage disequilibrium</td>
</tr>
<tr>
<td>LXR</td>
<td>liver X receptor</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
</tr>
<tr>
<td>MDR-1</td>
<td>multidrug resistance transporter 1</td>
</tr>
<tr>
<td>mIMCD-K2</td>
<td>mouse inner medullary collecting duct cell line</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>β-nicotinamide adenine dinucleotide, oxidized form</td>
</tr>
<tr>
<td>NADPH</td>
<td>β-nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGK</td>
<td>phosphoglycerate kinase</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PXR</td>
<td>pregnane X receptor</td>
</tr>
<tr>
<td>RAAS</td>
<td>renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-PCR</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SAME</td>
<td>apparent mineralocorticoid excess</td>
</tr>
<tr>
<td>SHR</td>
<td>spontaneously hypertensive rat</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
</tr>
<tr>
<td>SS</td>
<td>sodium sensitivity</td>
</tr>
<tr>
<td>-----</td>
<td>-------------------</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
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</table>
CHAPTER I: GENERAL INTRODUCTION
SUMMARY

CYP3A enzymes have established and possibly undefined roles in mammalian physiology in addition to their known functions as drug-metabolizing enzymes. CYP3A enzymes and associated transport proteins interact with physiologically important molecules. Genetic analyses suggest an evolutionary importance for human CYP3A isoforms. In this chapter, I review literature pertaining to CYP3A metabolism and physiology.

Herein I advance hypotheses concerning possible mechanisms of CYP3A action upon renal control of electrolyte balance and blood pressure. I also propose studies to address these ideas. These studies include a number of approaches and materials: human subjects, cellular and animal models, computational genetics, and meta-analyses of pre-existing studies. The conclusion offered by these studies is that roles for CYP3A enzymes in physiology remain intriguing, if puzzling, and that future studies would benefit from refining hypotheses and experimental methods.

INTRODUCTION

Cytochrome P450 Taxonomy

The cytochrome P450 (CYP) enzyme superfamily is an exceedingly diverse group with members found among organisms in every taxonomic kingdom (1). The CYPs are hemoproteins that have absorption maxima near 450 nm. CYP enzymes catalyze the oxidations and reductions of a wide array of substrates. CYPs are divided into roughly 237 numbered families on the basis of sequence homology:
family members share greater than 40% sequence similarity and subfamily members are more than 55% similar (2). Sixteen CYP families are found among mammals (3). In addition to their handling of endogenous molecules, members of the first three families are also known to metabolize exogenous, or xenobiotic, compounds— including therapeutic drugs (4). CYP1-3 proteins constitute the phase I drug-metabolizing enzymes, which catalyze the functionalization of their substrates via reactions (e.g., hydroxylation and demethylation) that increase aqueous solubility or introduce attachment sites for bulkier moieties to be added by phase II enzymes. CYP proteins are expressed in the liver and throughout the gastrointestinal tract, where the bulk of first-pass drug metabolism occurs (5). The generally non-CYP phase II enzymes— including glutathione S-transferases, sulfotransferases, and N-acetyltransferases— conjugate molecules to create products that are more soluble and more readily excreted by drug transporters (6). CYP activities may constitute a defense against foreign chemicals, but some have also been shown to generate harmful metabolites, such as those of benzo(a)pyrene and aflatoxin B1 (7).

**CYP3A enzymes**

The term “CYP3A” denotes family 3, subfamily A of the CYP superfamily. The CYP3A enzymes function in the endoplasmic reticulum and require an electron transport chain provided by NADPH-cytochrome P450 reductase (8). Among human CYP subfamilies, CYP3A is the most prominent— its members are the most highly expressed in the liver and intestines and they catalyze the metabolism of a tremendous variety of substrates, including more than half of currently marketed
drugs (9). The four human CYP3A isozymes, which are numbered 4, 5, 7, and 43, have extensively overlapping substrate specificities.

A gene cluster on human chromosome 7q21-q22.1 encodes the CYP3A enzymes; interspersed between the active genes are CYP3A pseudogenes, dormant remnants of duplication events (2, 10, 11) (Figure I-1). At least 6 mouse homologs reside in a syngeneic locus on mouse chromosome 5 (4, 12). All active human CYP3A genes have 13 exons. The evolutionary emergence of the CYP3A subfamily predates that of vertebrates, as CYP3-like genes are found even among the prevertebrate tunicates (13). CYP3A isoforms are expressed throughout the vertebrate subphylum (4, 14) and phylogenetic analyses predict that the common ancestor of all vertebrates bore a single CYP3A gene that evolved to generate modern CYP3A gene clusters in each species through species-specific gene-duplication and recombination events (14).

FIGURE I-1. Human CYP3A locus

The CYP3A metabolic unit

CYP3A enzymes can be considered to belong to a basic metabolic unit that also includes the nuclear hormone receptors pregnane X receptor (PXR) and retinoid X receptor (RXR) and the efflux transporter P-glycoprotein (P-gp), or multidrug resistance 1 transporter (MDR1). Levels of CYP3A and P-gp mRNAs are induced by
PXR/RXR heterodimers that bind to PXR response elements in CYP3A and MDR1 gene promoters (15, 16). PXR ligands include endogenous and xenobiotic compounds that are often CYP3A substrates and P-gp ligands (17). P-gp may alter the extent of CYP3A metabolism by pumping CYP3A substrates out of the cell, and CYP3A may control the activity of PXR and P-gp by metabolizing PXR/P-gp ligands into less avid products (18, 19). In summary, there is a homeostasis in this system that is controlled by interplay among its members. As will be discussed, the CYP3A/PXR/P-gp unit is at the nexus of a broad network of physiologic systems.

**CYP3A metabolism**

CYP3A enzymes participate in the oxidative, reductive, and peroxidative metabolism of a very diverse array of exogenous and endogenous substrates. Exogenous substrates include xenobiotic environmental chemicals, against which the CYP3A enzymes may form a line of defense. CYP3A enzymes metabolize a large number of therapeutic drugs and may be important in altering drug activity or in promoting drug clearance. Intriguing roles have emerged for CYP3A enzymes in endogenous processes such as steroidogenesis and steroid catabolism, lipid metabolism, and the processing of bile acids and vitamin D.

**Distribution of CYP3A expression**

Human CYP3A enzymes differ in their temporal and anatomic distributions. CYP3A7 expression is seen in utero but subsides by late infancy in most individuals (20). The CYP3A43 enzyme is limited in the distribution and magnitude of its expression and it
has low catalytic activity for CYP3A substrates (21, 22). Adult hepatic and intestinal CYP3A activity thus mainly reflects the contributions of CYP3A4 and CYP3A5.

CYP3A4 expression is variable but apparently universal: humans lacking CYP3A4 have yet to be identified. Genetic variations that may control the levels of CYP3A4 expression have been identified (23); the most studied of these is the CYP3A4*1B allele, reflecting an A-392G single-nucleotide polymorphism (SNP) in the CYP3A4 promoter that is associated with decreased CYP3A activity (24). In contrast to that of CYP3A4, CYP3A5 expression is clearly polymorphic - that is, it is absent from some people. Furthermore, the frequency of CYP3A5 deficiency varies from one population to the other (25). The major genetic causes of CYP3A5 deficiency are discussed below.

Among people who express CYP3A5, hepatic levels of CYP3A5 may be only 25% that of CYP3A4. But the hepatic aggregate CYP3A activity of CYP3A5 expressers may be exceed that of CYP3A5 non-expressers (25, 26). CYP3A4 and 5 may be co-expressed in extra-gastrointestinal sites such as the placenta (27). But CYP3A5 is also found in sites where CYP3A4 appears to be absent or undetectable. CYP3A5 is the predominant and perhaps exclusive 3A isoform in the kidney (28-30), including proximal and distal tubules and collecting ducts (21, 31); adrenal zona glomerulosa (21, 31); bronchial and alveolar respiratory epithelia (32); and anterior pituitary gland (33). Extra-gastrointestinal CYP3A expression is unlikely to contribute significantly to overall drug metabolism but may modulate peripheral toxicities and physiologic effects of exogenous and endogenous molecules (34, 35).
It is possible then that variance in some systemic and local adult CYP3A phenotypes reflects CYP3A5 polymorphism. For instance, CYP3A5 genotype and renal CYP3A5 expression may be associated with risk of nephrotoxic side effects due to CYP3A-substrate calcineurin inhibitors (tacrolimus and cyclosporin) (36-38). There has also been a suggestion that CYP3A5- and not CYP3A4- genotype modifies risk of a particular chronic nephritis (Balkan endemic nephropathy) whose likely etiologic agents include pro-toxic chemicals (e.g., ochratoxin A) that are activated by CYP3A enzymes (39, 40).

**CYP3A5 Genetic Variance**

Early studies of primarily Caucasian subjects suggested that the population-level impact of CYP3A5 expression would be small because the vast majority of subjects were clear non-expressers (41-43). However, expression frequency is much higher in non-white populations and generally exceeds half among black groups. In every human population studied so far, CYP3A5 expression variation mainly reflects the effect of a SNP, A6986G, in the third CYP3A5 intron that distinguishes the full-expresser allele (called *1) from a reduced-expresser allele (*3) (44). The A-to-G transition at this position functions as an alternative splice acceptor site that is preferred over the canonical exon 4 acceptor site; the frame-shifted variant mRNA predicts a truncated protein but the mRNA itself in unstable due to nonsense-mediated decay (45, 46). Two additional CYP3A5 SNPs, G14690A and 27131-32insT, respectively define the *6 and *7 alleles and are specific to black groups; the frequency of each allele is roughly 0.10 (25, 47). The *6 allele may cause splicing
defects that result in skipping of exon 7; the single-base insertion in exon 11 that defines *7 results in frame-shifted mRNA, a premature stop codon, and truncated protein (44, 45, 47).

**Ethnic differences in CYP3A5 allele frequencies**

The allelic frequency of CYP3A5*3- which is inversely proportional to the prevalence of CYP3A5 expression in a population- is roughly 0.27-0.30 among African-Americans (47), 0.60 among Southeast Asians (48), 0.73-0.76 among Chinese and Japanese (48-50), and 0.88-0.96 among Caucasians (21, 22, 26, 47, 51, 52). Accordingly, CYP3A5 protein levels are below limits of detection for Western blot in the majority of liver samples from Caucasians but are found in most samples from African-Americans (25). The *6 and *7 alleles, which are found almost exclusively among black groups, occur at respective frequencies of roughly 0.15 and 0.10. A summary of *3, *6, and *7 allele frequencies is shown in TABLE I-A. Information about lower-frequency CYP3A5 SNPs may be found elsewhere (23).

<table>
<thead>
<tr>
<th>TABLE I-A. CYP3A5 allele frequencies among populations</th>
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<tbody>
<tr>
<td><strong>Population</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Black</td>
</tr>
<tr>
<td>Afr.-American</td>
</tr>
<tr>
<td>Gabonese</td>
</tr>
<tr>
<td>Nigerian</td>
</tr>
<tr>
<td>Seychelles</td>
</tr>
<tr>
<td>Zimbabwean</td>
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<tr>
<td>Asian</td>
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<tr>
<td>Chinese</td>
</tr>
<tr>
<td>Japanese</td>
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<tr>
<td>Malay</td>
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</table>
### The Physiologic Impacts of CYP3A Enzymes

CYP3A enzymes play a critical role in the metabolism of bile acids. CYP3As belong to a minor pathway for the production of the primary bile acid cholate. CYP3A activity is the major route for clearance of the toxic secondary bile acid metabolite lithocholic acid (LCA). LCA is carcinogenic and may have a causative role in some cases of colon cancer. By preparing LCA for clearance, CYP3A enzymes limit its activation of prominent nuclear receptors such as farnesyl X receptor (FXR), the constitutive androstane receptor (CAR), and the vitamin D receptor.

### Oxysterol and lipid metabolism

The biologically-stable oxysterol 4β-hydroxycholesterol is produced by CYP3A enzyme activity (60, 61). It is a ligand for liver X receptor α (LXRα) (62). LXRα and β are nuclear receptors that are critical players in lipoprotein metabolism, cholesterol efflux, and glucose utilization. LXR activation is generally considered to have beneficial effects on energy balance: increased HDL levels and reverse cholesterol transport and increased glucose tolerance (63).
**Eicosanoids and renal function**

CYP3A enzymes are expressed in muscular arteries. In animal models, their production of epoxyeicosatrienoic acid (EET) metabolites of arachidonic acid appears to mediate potassium channel-dependent vasorelaxation (64-66). EETs modulate the effects of the epithelia sodium channel (ENaC) and angiotensin II on renal sodium excretion.

**Vitamin D, bone health, and cancer**

1α,25-dihydroxyvitamin D₃ induces CYP3A expression (67, 68), and CYP3A enzymes can inactivate 1α,25-(OH)₂D₃ through 24-hydroxylation and can thus potentially affect bone health. CYP3A inducers such as phenobarbital, ifosfamide, rifampin, and anticonvulsants have known potentials for reducing 1α,25-(OH)₂D₃ levels and causing rickets and osteomalacia (68-76). As mentioned, CYP3A enzymes inactivate LCA, a potent carcinogen that promotes its own detoxification through induction of CYP3A expression by activating the vitamin D receptor (VDR). Noted relationships between sunlight exposure and cancers of the colon, prostate, and breast (77) may reflect ultraviolet light activation of vitamin D, which enhances bile acid excretion (78), and the association of geographic latitude with CYP3A expression, discussed below.

**Steroid metabolism**

CYP3A enzymes exhibit broad specificity for steroids. The 3As have important roles in hydroxylation of testosterone (e.g., at the 2β, 6β, or 15β positions) and estrogen
(positions 4 and 16α) (79). It can be induced by various compounds including drugs, pesticides, and carcinogens, resulting in high CYP3A4 levels in liver and other tissues, including mammary. Oxidations of these steroids have incompletely defined effects on their bioactivities, but may be responsible for some of the associations between CYP3A activity and cancers of the prostate and breast. The particular reaction most pertinent to this review is 6β-hydroxylation (FIGURE I-2).

FIGURE I-2. CYP3A enzymes catalyze steroid 6β-hydroxylation

CYP3A-mediated 6β-hydroxylation is a metabolic pathway in vertebrates for steroids that include, in addition to androgens, the glucocorticoids cortisol and corticosterone, and the mineralocorticoid aldosterone. 6β-hydroxyglucocorticoid products of CYP3A-catalyzed reactions stimulate sodium transport across A6 toad kidney cells and induce sodium retention and increased creatinine clearance in rats (80-82).

CYP3A enzymes may modulate of A6 sodium transport through a mineralocorticoid receptor (MR)-mediated mechanism (83). Immunohistochemistry detects CYP3A proteins in rat kidney (80) and human distal tubules and collecting ducts (84), the principle sites at which corticosteroids stimulate sodium resorption. 6β-hydroxysteroids have been postulated to exert mineralocorticoid-like renal effects.
The findings of these studies present some confusion, however, as 6β-hydroxycortisol appears not to activate the MR but may antagonize it and appears not to act through the glucocorticoid receptor (GR). CYP3A activity, along with that of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), has been proposed to regulate glucocorticoid occupancy of MRs (83).

Human and animal data suggest the potential clinical importance of excess CYP3A activity. Renal CYP3A content and catalytic activity in the spontaneously hypertensive rat (SHR) are higher than in the normotensive Wistar-Kyoto rat (80, 85). Treatment of SHR with the CYP3A-selective inhibitor troleandomycin lowers BP (85). Several human diseases associated with elevated blood pressure, including Cushing’s syndrome (86) and pre-eclampsia (80), are characterized by increased urinary excretion of 6β-hydroxycortisol, a marker of in vivo CYP3A activity. A single study showed increased serum 6β-hydroxycortisol levels among essential hypertensives relative to normotensive individuals (87). Another demonstrated that a high-sodium diet caused an increase in urinary 6β-hydroxycortisol among sodium-sensitive African-American subjects in contrast to a decrease among those who were sodium-resistant (88). But it is not known whether elevated 6β-hydroxycortisol levels in these conditions truly reflect an increase in CYP3A activity or merely a spillover of increased cortisol levels into the CYP3A pathway, or whether 6β-hydroxysteroids play significant pathophysiologic roles in these diseases.
CYP3A5 and selective pressure

CYP3A5 expression by latitude

As discussed CYP3A5 expression is polymorphic. Why might CYP3A5 polymorphism have arisen and what might explain interpopulation differences in CYP3A5 expression? Certainly, the impact of CYP3A enzymes on modern pharmacology cannot be invoked here. Implicit in CYP3A5*3 allelic frequency data is the possibility of systematically higher prevalences of CYP3A5 expression among populations that have historically lived near the equator than among those from higher latitudes; data by Thompson, et al. bear this out. Using samples from the Human Genome Diversity Panel-Centre d’Etude du Polymorphisme Humain (HGDP-CEPH), these researchers show that CYP3A5*3 allele frequency correlates with latitude (Spearman’s \( \rho = 0.612; \ P < 0.0001 \)) (89). It is possible that this relationship is due to genetic drift (i.e., to sheer chance), but the finding that CYP3A5*3 frequency correlation with latitude in this study exceeded those of 99% of microsatellite alleles at genome-wide loci may point toward more than chance association. An unusually low degree of haplotype diversity among non-African samples (Coriell Cell Repositories) was noted at the CYP3A gene cluster- especially at CYP3A5- which would be consistent with selection at this locus (89-91).

Toxicologic pressure

Why might CYP3A5 expression frequency have declined with increasing latitude? Hypothetically, some deleterious effects of CYP3A5 expression may have progressively outweighed its benefits as humans moved away from the equator. The
diversity of CYP3A substrates makes plausible a variety of hypotheses. Certainly, the central role of the CYP3A/PXR/MDR1 system in xenobiotic metabolism suggests that chemical threats may have exerted pressure upon CYP3A expression. CYP3A enzymes have a demonstrated role in the activation of promutagenic heterocyclic amines (92-94). The procarcinogenic and proteratogenic aflatoxin B1 (AFB1) produced by *Aspergillus* fungal species is also activated by CYP3A enzymes, and there is an apparent CYP3A5 expresser allele dose relationship among Africans with biomarkers of AFB1 activation (95-97).

**Sunlight and vitamin D**

The involvement of CYP3A enzymes in vitamin D metabolism reasonably suggests an ability of CYP3A5 to modify the physiologic effects of sunlight exposure. During childhood, vitamin D deficiency can cause a number of rachitic skeletal features, including a shallow pelvis, which could negatively affect reproductive fitness (98). High CYP3A activity might have become detrimental at progressively polar latitudes; decreasing CYP3A5 activity would then parallel the loss of melanin that may enable ultraviolet light to form cholecalciferol in people living toward the poles (99, 100). CYP3A-mediated decreases in vitamin D may have had a secondary effect upon blood pressure and renal sodium balance—vitamin D is a negative regulator of the renin-angiotensin-aldosterone system (discussed below) (101) and vitamin D levels are inversely associated with blood pressure and the risk of hypertension (102, 103); vitamin D levels may be decreased in pre-eclamptic women (104). Lastly, vitamin D controls activity of the insulin-like growth factor (IGF) system, an important
determinant of birth weight (105, 106). Thus, CYP3A activity may play important roles in the modulation of reproductive fitness by vitamin D.

**Heat stress and sodium**

Ideas about vitamin D and sunlight exposure parallel those concerning the impacts of heat and sodium demand on African emigrants and their descendants. The modern human (*Homo sapiens sapiens*) emerged on the African continent 140,000 to 200,000 years ago, a figure based upon fossil records and mitochondrial DNA sequences (107, 108). The oldest known fossilized skeletons of modern humans to be found outside of the African continent—on the Arabian peninsula—date back to 100,000 years ago (109). Into the warm and humid African climate of that era arose a relatively hairless group with a tremendous capacity to sweat that aided in evaporative cooling but also imposed a risk for large losses of water and of sodium, the most critical ionic regulator of mammalian osmosis and extracellular volume. Low sodium content of tropical soil may have added to the risk of volume depletion and dehydration (110). It is likely that, among these early humans, those who had an enhanced ability to retain sodium and water enjoyed a significant survival advantage. Retention of these abilities at less stressful altitudes might have harmed reproductive success, as demonstrated by the ability of antidiuretic hormone (vasopressin), normally produced in response to dehydration, to cause preterm pregnant uterine contractions (111).

A role for CYP3A5 in the response to the threat of dehydration may be supported by the finding that human CYP3A expression, like that of aquaporin-2
(aqp2), increases in response to ambient hypertonicity. The promoters of the CYP3A4, 5, and 7 and aqp2 genes all contain tonicity-responsive enhancers (112).

**RAAS pathway**

Central to the physiological control of sodium and water balance is the renin-angiotensin-aldosterone system (RAAS). Classically, the renal juxtaglomerular apparatus (JGA) responds to a decrease in blood pressure or in the rate of sodium delivery to the kidney by releasing renin, which cleaves hepatically-secreted AGT to produce the angiotensin I (AT-I). The angiotensin-converting enzyme (ACE), which is found primarily in pulmonary vascular beds, processes AT-I into angiotensin II (AT-II). AT-II has a number of functions that include vasoconstriction and increasing aldosterone synthesis and secretion by adrenal zona glomerulosa cells. Aldosterone secretion leads to increased renal sodium and water reabsorption (113). Resetting of sodium and volume homeostasis causes feedback inhibition of renin release, decreasing AT-II production. The RAAS system was likely critical for early human survival. But when salt availability is not limited, excess RAAS activity may be detrimental- it has been implicated, for instance, in pre-eclampsia (114). Balance between sodium avidity’s positive and negative effects might thus have shifted with heat stress and sodium demand. Accordingly, linkage disequilibrium (LD) patterns at the AGT gene locus are suggestive of directional selection on this gene that correlates with latitude (115).
CYP3A5 A6986G covariance with hypertension gene candidates

The CYP3A5*3 allele (6986G), which is not physically linked to the AGT gene, may have faced similar pressures. In the study by Thompson, et al. the frequency of an AGT variant (M235) that predicts decreased plasma AGT levels correlated with latitude and with CYP3A5*3 frequency (Spearman $\rho=0.712$ and 0.680, respectively; both $P<0.0001$) (89). A separate group, led by Young, determined functional allele frequencies among the HGDP-CEPH panel for five HTN candidate genes and calculated standardized coefficients ($\beta$) for regression on absolute latitude. As shown in TABLE I-B, all but one allele were more strongly correlated with latitude than at least 96% of short tandem repeat markers (STRs) and control SNPs (116). Using data from Thompson, et al. (89), I calculated a $\beta$ coefficient of -0.58 for CYP3A5 A6986 ($P<0.0001$), indicating that each 10° increase in absolute latitude is associated with a CYP3A5*3 allele frequency increase of 0.09; the strength of its association with latitude places it among these HTN candidates.

<table>
<thead>
<tr>
<th>Allele</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT -6A</td>
<td>-0.91</td>
</tr>
<tr>
<td>GNB3 825T</td>
<td>-0.79</td>
</tr>
<tr>
<td>ENaCa -946G</td>
<td>-0.59</td>
</tr>
<tr>
<td>CYP3A5 A6986</td>
<td>-0.58</td>
</tr>
<tr>
<td>ENaCy -173G</td>
<td>-0.47</td>
</tr>
<tr>
<td>ADRB2 47A/79C</td>
<td>-0.40</td>
</tr>
<tr>
<td>AGT-217A</td>
<td>0.09</td>
</tr>
<tr>
<td>STRs and control SNPs</td>
<td>Mean: -0.03</td>
</tr>
<tr>
<td></td>
<td>SD: 0.21</td>
</tr>
<tr>
<td></td>
<td>Range: [-0.71, 0.71]</td>
</tr>
</tbody>
</table>
Direct interactions between CYP3A activity and the RAAS pathway have not been shown, but the CYP3A metabolic partner P-gp/MDR1 has been implicated in RAAS modulation. P-gp transports many CYP3A substrates— including aldosterone and cortisol (117, 118)—is expressed in the distal renal tubules and the adrenal cortex, and is an important component of the blood brain barrier in humans and in animal models (119-121). Subjects homozygous for an MDR1 reduced-expresser allele (3435T) gene show an exaggerated increase of serum aldosterone in response to angiotensin II infusion and fail to appropriately increase urinary sodium excretion in response to salt loading (122).

There is reason to suspect that the prehistoric CYP3A5 expression modulated physiologic control of sodium balance (25, 44). But the truth of this contention is likely unknowable. With respect to its modern importance, the most obvious hypothetical harm of high CYP3A5 expression is a risk of high blood pressure-sodium avidity in the setting of a modern abundance of dietary salt might lead to hypervolemia and/or hypertension.

Hypertension itself is a major continuous risk factor for disability and death related to disease of the coronary vessels and peripheral vasculature, cerebrovascular accidents, and renal disease. It is perhaps worth noting that not only are people of African descent the most frequent CYP3A5 expressers but also that African-Americans have the highest global rates of sodium sensitivity and HTN (123, 124). Both hypertensive and normotensive blacks demonstrate, on average, lower plasma renin levels than whites (125). This consistent finding may indicate a
decreased capacity for renal sodium excretion among African-Americans or an increased tendency toward sodium reabsorption by unidentified mechanisms.

Potentially related to increased rates of hypertension among African-Americans is a higher frequency of pregnancies that result in low-birth weight babies. A number of epidemiologic studies demonstrate an association between low birth weight or prematurity and the risk of adult hypertension.

**UNIFYING HYPOTHESIS**

Some of the effects of 6β-hydroxycorticosterone may mimic classic mineralocorticoid activity, but it appears to activate sodium flux primarily through a distinct non-MR/non-GR A6 cell binding site (81). Subsequent studies suggest that such a binding site may be 11β-HSD2 (126, 127), an NADH-dependent enzyme that limits activation of MR by cortisol and corticosterone in aldosterone-sensitive epithelia by catalyzing their conversion to their respective 11-oxidized metabolites cortisone and 11-dehydrocorticosterone, which are weaker agonists for MR (Figure I-3).
Genetic 11β-HSD2 deficiency causes the syndrome of apparent mineralocorticoid excess (AME), so named because its features of hypertension and hypokalemia would suggest high aldosterone levels. To the contrary, aldosterone and renin levels in this condition are low, indicating a sodium-replete hypervolemia. Glycyrrhetinic acid derivatives such as carbenoxolone inhibit 11β-HSD2 and cause an AME-like pseudoaldosteronism. The existence of an 11β-HSD2 knockout mouse model that exhibits hypertension, hyporeninemia, and low birth weight reinforces the physiologic importance of the enzyme.

It may be speculated that similarities between 11β-HSD2 deficiency and the syndromic presentation of sodium sensitivity, decreased plasma renin, and low birth weight among African-Americans as a group is suggestive of a possible role for reduced 11β-HSD2 in this phenomenon. I have hypothesized that CYP3A effects in
mineralocorticoid-sensitive tissues reflect 6β-hydroxysteroid inhibition of 11β-HSD2 (FIGURE I-4). The significance of the proposed studies lies in their potential to legitimize or disprove a causative role for CYP3A activity in vertebrate renal physiology and to unmask possible molecular mechanisms underlying their effects. The gap in the literature that these studies may remedy is the demonstration that BP and other physiological effects can be specifically attributed to CYP3A activity and are not simply the result of association due to confounding. The particular promise of the CYP3A system lies not only in their possible etiologic role in HTN but also in their capacity to metabolize medications that include antihypertensives such as the calcium-channel blockers; thus, CYP3A activity may not only influence the susceptibility to HTN but also the response to some pharmacologic therapies. Because of this potential CYP3A “double effect”, this system may be a promising target for novel drug therapy.
FIGURE I-4. Proposed mechanism of $6\beta$-hydroxysteroid effects on renal sodium transport.
CHAPTER II: CYP3A5 GENOTYPE PREDICTS RENAL CYP3A ACTIVITY AND BLOOD PRESSURE IN HEALTHY ADULTS

(Chapter II contains material originally printed in J Appl Physiol 2003; 95:1297-1300. It is used with permission of the American Physiological Society.)
SUMMARY

A single nucleotide polymorphism (SNP), A6986G, in the cytochrome P450 3A5 (CYP3A5) gene distinguishes an expressor (*1) and a reduced-expressor (*3) allele and largely predicts hepatic and intestinal CYP3A5 content. CYP3A5 is the prevailing CYP3A isoform in the kidney, where CYP3A activity has been proposed to affect blood pressure (BP) by altering sodium transport. This chapter reports that among renal microsomes from 21 organ donors, those from *1/*3 individuals had at least 8-fold higher mean CYP3A5 content and 18-fold higher mean CYP3A catalytic activity than did microsomes from *3/*3 individuals (p=0.0001 and p=0.0137, respectively). Among a group of 25 healthy African-American adults, there were significant associations between the A6986G SNP and systolic BP (SBP; p=0.0007), mean arterial pressure (MAP; p=0.0075), and creatinine clearance (CrCl; p=0.0035). These associations remained significant when sex, age, and body mass index (BMI) were taken into account. The mean SBP of homozygous CYP3A5 expressors (*1/*1) exceeded that of homozygous non-expressors (*3/*3) by 19.3 mmHg. It is speculated whether a high CYP3A5 expressor allele frequency among African-Americans may contribute to a high prevalence of sodium-sensitive hypertension in this population.

INTRODUCTION

Cytochrome P450 3A (CYP3A) homologs are variably expressed in human and rat liver (25, 128), kidney (28, 80), adrenal gland (21), and anterior pituitary gland (33).
The physiologic effects of CYP3A enzyme activity have not been clearly defined, but some observations support a role in the control of blood pressure (BP). CYP3A content and catalytic activity in the kidneys of spontaneously hypertensive rats (SHR) are higher than in normotensive Wistar-Kyoto rats (80, 85). Treatment of SHR with the CYP3A inhibitor troleandomycin lowers BP (85). Several human diseases associated with elevated BP, including Cushing’s syndrome (129) and pre-eclampsia (80), are characterized by increased urinary excretion of $6\beta$-hydroxycortisol, a marker of in vivo CYP3A activity (85, 130). Plasma levels of $6\beta$-hydroxycortisol have been reported to be elevated in essential hypertensives (87).

A role for CYP3A enzymes in blood pressure control appears scientifically plausible. CYP3A-mediated $6\beta$-hydroxylation is a minor metabolic pathway for steroids, including the glucocorticoids cortisol and corticosterone. $6\beta$-hydroxysteroid products of CYP3A-catalyzed reactions stimulate sodium transport across A6 toad kidney cells and sodium retention in rats (80-82). Previous evidence indicates $6\beta$-hydroxycorticosterone modulation of A6 cell sodium transport by acting through an unidentified binding site (80, 81). Consistent with data from rat kidney (80, 131), immunostaining for CYP3A highlights human distal tubules and collecting ducts (84), the principle sites at which aldosterone stimulates sodium reabsorption. Renal CYP3A activity, along with that of $11\beta$-hydroxysteroid dehydrogenase type 2, has been proposed to regulate glucocorticoid occupancy of the mineralocorticoid receptor (MR) (83).

It has been established that, among all CYP3A isoforms, the expression of CYP3A5 prevails in the human kidney (28, 37). The discovery of genetic variants
underlying polymorphic CYP3A5 activity has made possible non-invasive studies of the contribution of polymorphic CYP3A5 expression to human renal CYP3A activity and blood pressure. CYP3A5 expression in liver (25) and intestine (26) is largely determined by the A6986G SNP that distinguishes the CYP3A5*1 ("expressor") allele from the *3 (a "reduced-expressor") allele. This study reports that A6986G predicts renal CYP3A5 activity among mostly Caucasian organ donors and BP and CrCl among a group of healthy young African-American adults.

**METHODS**

All studies were approved by the University of North Carolina (UNC) Committee on the Protection of Human Research Subjects.

**Renal microsome studies:** CYP3A catalytic activity and CYP3A5 immunoreactivity were assayed in renal tissue samples from 21 organ donors- 17 were Caucasian, one was Hispanic, and three were of unknown ethnicity. All methods were described previously (26). CYP3A5 genotypes were determined by direct genomic DNA sequencing (see below). Microsomes were isolated by differential centrifugation. CYP3A5 content in 50 µg of microsomes was measured by Western blot with a CYP3A5-specific antibody (BD Gentest; Woburn, MA); undetectable bands were assigned a value of 0.25 pmol/mg, the limit of quantitation (LOQ; Figure 1A). Midazolam (MDZ) 1'-hydroxylation was measured after a 15-minute incubation of 200 µg protein with 8 µM MDZ; microsomes with undetectable activity were given the LOQ value of 0.04 pmol/min/mg.
**Human subjects:** 89 unrelated self-identified healthy African-American volunteers between 18 and 65 years of age were genotyped for the CYP3A5 *3* allele using previously-described methods, as below (25). For all subjects, in-person informed consent was obtained.

**Clinical measurements:** A subset of this group, consisting of 25 healthy individuals (age range: 18-52 years) who volunteered to be screened for a pharmacogenetic study, were tested for the ancillary hypothesis of CYP3A5 genotype association with blood pressure and creatinine clearance. Subjects were brought to the UNC General Clinical Research Center (GCRC), where they were examined and donated blood and urine samples. GCRC nurses who were blind to subject genotypes and medical histories performed and recorded all clinical measurements. Creatinine clearance was calculated by the Cockcroft-Gault equation (132).

**Genomic DNA collection and isolation:** Subjects were instructed to swish vigorously with 10 mL Scope® mouthwash for 1 minute and expel the rinse into 50-mL conical vials. Samples were placed at room temperature for a maximum of 48 hours until further processing. DNA was extracted from rinse samples with the use of either a QIAamp DNA Blood Mini Kit (Qiagen, Inc.; Valencia, CA) or a Puregene DNA Isolation Kit for mouthwash samples (Gentra Systems) according to the manufacturer’s instructions. Total DNA concentrations were determined by spectrophotometry at 260 nm.
**CYP3A5 genotype determinations:** The *CYP3A5* variant was identified by polymerase chain reaction (PCR). The forward primer for this reaction was 5'-CCTGCCTTCAATTTCACCTAG-3', which anneals at position 22719 (Genbank no. AC005020); the reverse primer was 5'-GCAATGTTAGGAAGGAGGGCT-3', which anneals at position 24161 (Genbank no. AC005020). PCR was performed as follows: 50 ng DNA was initially denatured at 92°C for 5 minutes followed by 35 cycles of denaturation at 92°C for 30 seconds, annealing at 61°C for 30 seconds, and synthesis at 72°C for 2 minutes. The final elongation was carried out for 5 minutes at 72°C. The unincorporated nucleotides and primers were removed as described above. Sequencing was carried out on an ABI Prism 3700 Automated Sequencer. The nested primers used to sequence were 5'-TAATATTCTTTTGGATAATG-3' (f) and 5'-CATTCTTTCACCTAGCAGCTGATTC-3' (r). Sequences were assembled using the Polyphred program (University of Washington, Seattle), which automatically detects the presence of heterozygous SNP substitutions by fluorescence-based sequencing of PCR products.

**Statistical analyses:** Microsomal CYP3A5 content and MDZ 1'-hydroxylation were compared between genotypes with the Wilcoxon signed-rank test for non-parametric distributions. Differences between human subject genotype groups were assessed by ANOVA and Fisher’s exact test for continuous and categorical variables, respectively.
RESULTS

**CYP3A5 genotype predicts renal CYP3A activity:** The average CYP3A5 content (1.98 pmol/mg; n=5) of microsomes from *1/*3 kidneys exceeded the average of those from *3/*3 kidneys (n=16; p=0.0001), which was at or below the limit of quantitation (Figure I-IA). None of the kidneys were *1/*1. CYP3A4 was not detected with a CYP3A4-specific antibody in any of the microsomal preparations. Mean microsomal CYP3A activity, reflected by MDZ 1'-hydroxylation, was 18-fold higher in *1/*3 kidney microsomes (8.04 pmol/min/mg) than in those from *3/*3 kidneys (0.43 pmol/min/mg; p=0.0137; Figure I-IB). The *1/*3 outlier indicated by arrows in Figure I also bore a *7 allele and was probably a compound heterozygote. Removal of this outlier slightly lowered p values.

**CYP3A5 genotype predicts blood pressure:** The *CYP3A5 A6986* (*1 allele) frequency among the 89 African-Americans was 0.7. Among the 25-subject subset (TABLE II-A), *CYP3A5* genotype associated with seated systolic BP (SBP), mean arterial pressure (MAP), the product of SBP and heart rate (SBP*HR, an indicator of left-ventricular oxygen consumption), and Cockcroft-Gault creatinine clearance (CrCl). The *1/*1 group averaged the highest value for each measure. Average *1/*1 SBP exceeded that of the *3/*3 group by 19.3 mm Hg (Figure I-II) and a gene-dose effect was apparent. *CYP3A5* genotype associated significantly with combined BP strata (p=0.0048; TABLE II-A) from the Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC-
VI; N=Optimal or Normal [SBP <130 and DBP <85]; H=High-Normal or higher [SBP ≥130 or DBP ≥85]) (133).

Sex-specific analysis detected CYP3A5 genotype associations with SBP, MAP, and estimated CrCl among females (TABLE II-A); similar trends were noted among the smaller sample of males, but these did not reach statistical significance. In multiple regression analyses, sex and genotype accounted for 70% of the variability in SBP. When compared to non-*1/*1 females, *1/*1 males showed more than a 20-fold increased risk of high-normal or higher BP (p=0.0002).

Subject age, averaging roughly 25 years, did not vary between genotype groups. Age and body mass index (BMI) effects upon BP were non-significant. Genotype association with BMI, diastolic blood pressure (DBP), pulse pressure (PP), and HR did not reach statistical significance.

**DISCUSSION**

The CYP3A5*1 allele associates with CYP3A5 expression in the human kidney- a finding which was previously reported for liver and intestine. However, in the liver and intestine, there is also substantial CYP3A4 expression (25, 26). As a result, hepatic and intestinal aggregate CYP3A activities associate only weakly with the CYP3A5*1 allele. In contrast, CYP3A4 was not detected in renal microsomes, and mean CYP3A activity differed markedly between CYP3A5 genotype groups.

The preliminary finding of CYP3A5 genotype association with resting BP among healthy adults may be consistent with a role for CYP3A enzymes in BP control. Young adults with supernormal BP have an increased long-term risk of
death due to cardiovascular and coronary heart disease (134); the identification of common genetic polymorphisms relevant to BP control is thus an important line of investigation. If shown true, this conjecture might portend a role for inhibition of CYP3A5 activity or expression in the treatment of some forms of hypertension.

The *CYP3A5 A6986* allele frequency among this cohort of African-Americans agrees roughly with a previous report (47). This frequency exceeds those among all other ethnic populations studied to date (25, 47). Inter-ethnic differences in the prevalence of sodium-sensitivity (124) parallel those of hypertension (123), with African-Americans having the highest global prevalence of each. A possible link between CYP3A5 activity and the high prevalence of sodium-sensitive hypertension among African Americans may merit further study.
FIGURE II-1. CYP3A activity and CYP3A5 content in *1/*3 and *3/*3 renal microsomes from 21 organ donors.
A: CYP3A5 content differs between *1/*3 and *3/*3 renal microsomes (p=0.0001). B: MDZ 1'-hydroxylation differs between *1/*3 and *3/*3 renal microsomes (p=0.0137). Analysis of DNA from the *1/*3 outlier, indicated by arrows, revealed the inactivating *7 variant. Bars show average values.
FIGURE II-2. Seated systolic blood pressure among 25 healthy African American adults by CYP3A5 genotype.
SBP varies among genotypic groups (p=0.0007). Nurses blinded to subject genotypes performed all measurements. Bars show average values.
<table>
<thead>
<tr>
<th></th>
<th>*1/*1</th>
<th>*1/*3</th>
<th>*3/*3</th>
<th>p</th>
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<tbody>
<tr>
<td><strong>All Subjects</strong></td>
<td>n=9</td>
<td>n=9</td>
<td>n=7</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>24.5 (10.7)</td>
<td>25.0 (8.0)</td>
<td>25.0 (8.5)</td>
<td>0.9933</td>
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<tr>
<td>BMI (kg/m^2)</td>
<td>30.3 (8.1)</td>
<td>23.9 (4.6)</td>
<td>26.2 (2.8)</td>
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<td>SBP (mm Hg)</td>
<td>136.0 (7.9)</td>
<td>121.6 (8.4)</td>
<td>116.7 (11.0)</td>
<td>0.0007</td>
</tr>
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<td>DBP (mm Hg)</td>
<td>71.4 (5.1)</td>
<td>68.1 (10.1)</td>
<td>63.3 (6.5)</td>
<td>0.1283</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>92.9 (3.8)</td>
<td>85.9 (9.0)</td>
<td>81.1 (6.5)</td>
<td>0.0075</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>64.6 (10.3)</td>
<td>53.4 (7.0)</td>
<td>53.4 (10.6)</td>
<td>0.0309</td>
</tr>
<tr>
<td>HR (min^{-1})</td>
<td>78.9 (15.3)</td>
<td>73.9 (9.0)</td>
<td>70.6 (8.3)</td>
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</tr>
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<td>SBP<em>HR (mm Hg</em>min^{-1})</td>
<td>10743.1 (2253.3)</td>
<td>8948.6 (1006.2)</td>
<td>8189.6 (810.8)</td>
<td>0.0088</td>
</tr>
<tr>
<td>JNC-VI stratum (N/H)</td>
<td>2/7</td>
<td>8/1</td>
<td>6/1</td>
<td>0.0048</td>
</tr>
<tr>
<td>CrCl (ml/min)</td>
<td>147.2 (30.9)</td>
<td>106.9 (23.7)</td>
<td>110.4 (10.5)</td>
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<tr>
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<td>n=6</td>
<td>n=5</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>20.8 (2.8)</td>
<td>20.8 (2.6)</td>
<td>25.8 (9.6)</td>
<td>0.3426</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>130.8 (6.2)</td>
<td>117.5 (3.9)</td>
<td>112.2 (9.1)</td>
<td>0.0039</td>
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<tr>
<td>MAP (mm Hg)</td>
<td>91.9 (3.8)</td>
<td>82.5 (2.9)</td>
<td>80.2 (7.6)</td>
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<td>CrCl (ml/min)</td>
<td>156.9 (16.6)</td>
<td>114.7 (24.3)</td>
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<td><strong>Males</strong></td>
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<td>n=2</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>27.6 (14.0)</td>
<td>33.3 (9.1)</td>
<td>23.0 (7.1)</td>
<td>0.6439</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>140.2 (6.8)</td>
<td>129.7 (9.8)</td>
<td>128.0 (5.7)</td>
<td>0.1355</td>
</tr>
<tr>
<td>CrCl (ml/min)</td>
<td>139.5 (39.2)</td>
<td>91.4 (15.4)</td>
<td>109.0 (14.9)</td>
<td>0.1661</td>
</tr>
</tbody>
</table>

Values are shown as mean (SD). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; mean arterial pressure (MAP); PP, pulse pressure; HR, heart rate; CrCl, creatinine clearance; JNC-VI, combined BP stratum from the Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure; N, Optimal or Normal (SBP <130 and DBP <85); H, High-Normal or higher (SBP ≥130 or DBP ≥85).
CHAPTER III: CYP3A5 HAPLOTYPE RECONSTRUCTION
SUMMARY

Cytochrome P450 3A5 (CYP3A5) is a member of the CYP3A enzyme subfamily, which catalyzes the metabolism of the majority of marketed drugs. CYP3A5 is expressed polymorphically between individuals, and the frequency of expression varies between ethnic groups. An individual's ability to express CYP3A5 is defined by known single nucleotide polymorphisms (SNPs) in the CYP3A5 gene. The A6986G SNP distinguishes the CYP3A5*1 (or “full-expresser”) allele from the dysfunctional variant *3 allele, the major expression-determining allele common to all populations. Considerable interest has arisen in the potential of CYP3A5 genotype as a predictor of drug metabolism and as a disease risk factor. Among blacks, the predictive power of the *3 allele for CYP3A5 expression may lowered by the presence in this group of two additional alleles, called *6 and *7. In this chapter, an expectation-maximization (EM) algorithm is used to reconstruct CYP3A5 haplotypes among a sample of African-Americans who were genotyped for the three dysfunctional alleles. This method deduced the presence of four CYP3A5 haplotypes: the full-expresser haplotype and three single-variant haplotypes. This finding may obviate the need for future molecular CYP3A5 haplotyping assays. Statistical haplotype reconstruction may restore the predictiveness of CYP3A5 SNPs and lower genotyping costs.

INTRODUCTION

The CYP3A subfamily consists of CYP3A4 and CYP3A5 in adults. While CYP3A4 expression is universal, CYP3A5 is expressed by a minority of individuals in most populations but by a majority of blacks of African descent (25, 53, 55). Interpersonal
variance in CYP3A5 activity may be clinically important. There is a potential for CYP3A5 activity to predict an individual's ability to metabolize drugs. For instance, a number of studies have demonstrated a higher dose requirements for the immunosuppressant CYP3A substrate tacrolimus among CYP3A5-expressing organ recipients, compared to non-expressers (36, 38, 135-137). CYP3A5 and CYP3A activity may additionally have roles in the modification of disease risk, as suggested by CYP3A metabolism of estrogens (138-144), androgens (145-148), and corticosteroids (86, 149-152). There have been intriguing but inconstant associations of CYP3A5 genotype with cancers of the prostate, lung, and breast (153-158); hematological malignancies (159, 160); early puberty (161); and blood pressure (30, 54, 162-173).

The genetic basis for the bulk of CYP3A5 polymorphism was first described by Kuehl, et al. (25), who discovered an A-to-G transition (A6986G) that activates an alternative splice-acceptor site in the third intron of the CYP3A5 gene. The presence of guanine at this position defines the *3 allele, a dysfunctional variant that produces negligible amounts of functional enzyme, as compared to the functional *1 allele.

Inter-group differences in the prevalence of CYP3A5 expression mainly reflect variation in the frequency of *3, which is 0.85-0.95 among whites (25, 55), 0.76-0.78 among Chinese (48, 56) and 0.2-0.3 among blacks (25, 30, 55). The *3 allele predicts whether CYP3A5 is expressed in the liver (25), intestine (26), and kidney (30). But CYP3A5 genotype at the 6986 site may have lower predictive power for blacks than for other groups.
Kuehl, et al. and Hustert, et al. identified two other low-expressor alleles, *6 and *7, that respectively reflect G14690A and 27131-32insT SNPs. These are specific to blacks at frequencies of roughly 0.15 for *6 and 0.10 for *7 (25, 47). The G14690A SNP causes aberrant splicing, while 27131-32insT causes a frame shift resulting in premature termination of transcription. Each of the resulting alleles has been shown to decrease CYP3A5 protein expression (25, 30, 45, 47).

The presence of multiple dysfunctional SNPs in a particular gene can lower the power of the genotype at any individual locus in the gene to predict phenotype. In such situations, it may be important to determine haplotype. Experimental methods for directly determining haplotypes, such as the creation of yeast artificial chromosomes (174) or the creation of mouse-human hybrid cell lines (175), can be too technically difficult, laborious, or expensive for large-scale studies. While a subject’s haplotype may also be deduced from genotypes of the subject’s family members (176), collection of these additional samples may be impractical at least. It may also be possible to reconstruct haplotypes by using statistical methods to infer phase from genotypes at linked loci. One of the most popular approaches for this is based upon maximum likelihood and uses the expectation-maximization (EM) algorithm (177-179), which can produce accurate estimates of haplotype frequencies for a wide range of settings (180).

The EM algorithm was used to resolve CYP3A5 genotypes at the 6986, 14690, and 27131 positions among a group of African-Americans into four haplotypes. The implications of CYP3A5 haplotyping for CYP3A phenotype prediction will be discussed.
METHODS

Human subjects: 117 unrelated self-identified African-American adults were genotyped for the CYP3A5 *3, *6, and *7 SNPs. In-person informed consent was obtained for all subjects. The study was approved by the Institutional Review Board of the University of North Carolina School of Medicine. Genotypes were obtained by genomic DNA sequencing, as described (25).

CYP3A5 genotype determinations: CYP3A5 exon 3B was amplified for identification of the CYP3A5*3 variant by polymerase chain reaction (PCR). The forward primer for this reaction was 5'-CCTGCCTTCAATTTCCTG-3', which anneals at position 22719 (Genbank no. AC005020); the reverse primer was 5'-GCAATGTAGGAAGGAGGGCT-3', which anneals at position 24161 (Genbank no. AC005020). PCR was performed as follows: 50 ng DNA was initially denatured at 92°C for 5 minutes followed by 35 cycles of denaturation at 92°C for 30 seconds, annealing at 61°C for 30 seconds, and synthesis at 72°C for 2 minutes. The final elongation was carried out for 5 minutes at 72°C. The unincorporated nucleotides and primers were removed as described above. Sequencing was carried out on an ABI Prism 3700 Automated Sequencer. The nested primers used for sequencing were 5'-TAATATTCTTTTGATAATG-3' (f) and 5'-CATTCTTTCACTAGCCTGGTTC-3' (r). The primers used to amplify the exon 7 deletion in CYP3A5*6 were 5'-GGTCATTGCTGTCTCCAACC-3' (f) and 5'-TCAAAACTGGGGGTAGGAATGT-3' (r). The PCR was performed as above with an annealing temperature of 59°C. Sequencing was carried out using nested primers 5'-TATGACTGGGCTCCTTGGACC-
3' (f) and 5'-TGGAATTGTACCTTTTAAGTGGA-3' (r). PCR for the *7 insertion was performed with the following primers: 5'-AAATACTTCACGAATACTATGATCA-3' (f) and 5'-CAGGGACATAATTGATTATCTTTG-3' (r). Sequences were assembled using the Polyphred program (University of Washington, Seattle), which automatically detects the presence of heterozygous SNP substitutions by fluorescence-based sequencing of PCR products.

**Haplotype reconstruction:** CYP3A5 genotypes at each locus were assessed for Hardy-Weinberg equilibrium and were subjected to the EM algorithm with Arlequin 2.0 software (181). The software was programmed to allow 300 initial conditions for haplotype frequency estimation, 200 initial conditions for the linkage disequilibrium test with 20,000 permutations, and 200 initial conditions for the bootstrap procedure with 100,000 replicates.

**RESULTS**

The observed CYP3A5*3, *6, and *7 allele frequencies among 117 African-Americans were 0.329, 0.150, and 0.103, respectively; these are consistent with previous studies (25, 47). Complete linkage disequilibrium (D' =1.0) was observed between each pair of loci. The frequencies and estimated standard deviations of haplotypes deduced by EM are shown in Figure III-I. Of the 8 possible haplotypes that might be constructed from the 3 biallelic CYP3A5 loci (2^3 = 8), only the full-expresser (*1-*1-*1) and the three singly-variant haplotypes are detected. The deduced frequency of the full-expresser haplotype (*1-*1-*1) is 0.426 (0.035). The
distributions of diplotypes (haplotype combinations) among CYP3A5 expression phenotypes are shown in FIGURE III-1.

**TABLE III-A. CYP3A5 allele frequencies among 117 African-Americans**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
<th>Std. Error</th>
<th>Genotype (N)</th>
<th>HWE χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>*3 (6986G)</td>
<td>0.329</td>
<td>0.043</td>
<td>54 49 14</td>
<td>0.311</td>
<td>0.675</td>
</tr>
<tr>
<td>*6 (14690A)</td>
<td>0.150</td>
<td>0.033</td>
<td>83 33 1</td>
<td>1.382</td>
<td>0.240</td>
</tr>
<tr>
<td>*7 (27131insT)</td>
<td>0.103</td>
<td>0.028</td>
<td>94 22 1</td>
<td>0.054</td>
<td>0.817</td>
</tr>
</tbody>
</table>

**TABLE III-B. CYP3A5 haplotype frequencies among African-Americans**

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Constituent Alleles</th>
<th>Frequency</th>
<th>Std. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1-*1-*1</td>
<td>6986A-14690G-27131wt</td>
<td>0.426</td>
<td>0.035</td>
</tr>
<tr>
<td>*3-*1-*1</td>
<td>6986G-14690G-27131wt</td>
<td>0.327</td>
<td>0.033</td>
</tr>
<tr>
<td>*1-*6-*1</td>
<td>6986A-14690A-27131wt</td>
<td>0.158</td>
<td>0.026</td>
</tr>
<tr>
<td>*1-*1-*7</td>
<td>6986A-14690G-27131insT</td>
<td>0.089</td>
<td>0.020</td>
</tr>
</tbody>
</table>
FIGURE III-1. Reconstructed CYP3A5 diplotype and phenotype frequencies.
The frequency of each diplotype deduced by EM is indicated; the proportion of each diplotype among its respective functional category is shown in parentheses. H: high CYP3A5 activity; I: intermediate activity; L: low activity

The presence of multiple unphased causative SNPs threatens to complicate attempts to associate gene function with phenotype. It is hoped that haplotyping can improve the power to detect gene-phenotype associations. Using the data presented in Chapter II, comparison of power versus sample size for homozygous expresser and nonexpresser groups based upon genotype at 6986 or haplotype shows mixed results (FIGURE III-2). Use of CYP3A5 haplotype is predicted to slightly reduce power at a given sample size for systolic blood pressure, but it is predicted to greatly increase power to detect differences in body mass index or creatinine clearance.
FIGURE III-2. Power versus sample size for CYP3A5 genotype and haplotype. Curves compare power as a function of sample size between genotype at CYP3A5 A6986 (bold line) and CYP3A5 haplotype (light line). **A.** systolic blood pressure. **B.** body mass index. **C.** creatinine clearance.

**DISCUSSION**

If taken to be representative of African-Americans broadly, these data would imply that CYP3A5 expressers are roughly 67% of this group, by the Hardy-Weinberg equilibrium equation. This figure exceeds estimated proportions of expressers among other groups- 30-40% among Asian groups, 10-20% among whites- but is much lower than estimates of roughly 90% based upon the *3 allele in this and other studies. Among 6986 *1/*1 homozygotes, less than 25% would have two truly
functional CYP3A5 genes, nearly 75% would have only one, and 3% would be non-expressers. Of those deemed to be expressers based upon 6986 genotype (*1/*1 or *1/*3) nearly 25% would be true non-expressers. Less than 40% of true non-expressers would be identified on the basis of a *3/*3 genotype. In a hypothetical scenario of CYP3A5 expression perfectly predicting a phenotype, the presence of true non-expressers in these proportions among presumed expressers would lower predictiveness by 25%. In addition to position 6986, the determination of genotypes 14690 and 27131 and the haplotype combinations among these sites is likely to be important in evaluating associations between CYP3A5 expression and various phenotypes among black subjects.

African-American CYP3A5 haplotypes among these sites can be inferred from population data with the E-M algorithm. This computational approach may improve the predictive power of CYP3A5 genotype and likely renders molecular haplotyping methods unnecessary. The apparent lack of recombination between these positions is perhaps not surprising given the relatively short distances between them (<21 kb). Confirmation of non-recombination among these sites may also be used to lower the costs of CYP3A5 genotyping by allowing analysis of one locus at a time and stopping once two variant alleles have been identified for a particular subject, thus reducing the number of reactions that must be performed to accurately predict an individual’s phenotype.
CHAPTER IV: IN VITRO STUDIES OF 6β-HYDROXYCORTICOSTERONE
SUMMARY

Excessive sodium reabsorption by the kidney may cause increased plasma volume and a resultant hypertension. Sodium sensitivity, independently and as a cause of hypertension, is associated with an increased risk of premature death. Cytochrome P450 3A (CYP3A) enzymes have been proposed to alter renal sodium reabsorption by catalyzing the conversion of glucocorticoids such as cortisol or corticosterone to 6β-hydroxylated metabolites with altered activity. The mechanisms by which these metabolites act in the kidney have not previously been identified.

This chapter details an exploration of the effects of corticosterone and 6β-hydroxycorticosterone on gene expression in cultured renal collecting duct cells and an evaluation of the hypothesis that 6β-hydroxycorticosterone exhibits physiologically relevant inhibition of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which prevents inappropriate activation of the mineralocorticoid receptor (MR) by glucocorticoids. The results show that, on its own, 6β-hydroxycorticosterone does not markedly influence epithelial sodium channel α-subunit (ENaC-α) gene expression but increases ENaC-α mRNA levels when it is applied in addition to B, its parent compound. It is suggested that this activity reflects the ability of 6β-hydroxycorticosterone to inhibit inactivation of corticosterone by the 11β-HSD2 enzyme- a contention which is supported by enzyme kinetic data that show that 6β-hydroxycorticosterone limits corticosterone conversion in a manner consistent with reversible, competitive inhibition.
Exploration of this phenomenon may provide insights into the renal control of electrolyte balance and blood pressure and may suggest new pharmacologic targets for antihypertensive therapy and the control of sodium sensitivity.

INTRODUCTION

CYP3A enzymes catalyze the oxidative metabolism of a wide variety of lipophilic molecules, including steroids. The results of animal and in vitro studies indicate that CYP3A-catalyzed 6β-hydroxylation of corticosterone in the kidney can alter renal sodium transport. 6β-hydroxycorticosterone increases sodium transport across cultured A6 Xenopus laevis toad kidney cells and induces sodium retention in spontaneously hypertensive rats (SHR). Some effects of 6β-hydroxycorticosterone may mimic classic mineralocorticoid activity, but 6β-hydroxycorticosterone appears to act through a binding site for corticosterone that is distinct from MR or the glucocorticoid receptor (GR) (81). The identity of this binding site remains unknown.

Subsequent studies demonstrate that some high-affinity binding sites for corticosterone in mammalian renal collecting duct consist of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (126, 127). 11β-HSD2 is an NAD⁺-dependent enzyme that limits glucocorticoid activation of MR in aldosterone-sensitive epithelia by catalyzing the conversion of cortisol and corticosterone to their respective 11-oxidized metabolites (cortisone and 11-dehydrocorticosterone), which are weaker MR agonists.

Human genetic absence of 11β-HSD2, encoded by the HSD11B2 gene, causes the syndrome of apparent mineralocorticoid excess (AME), so named
because its classic features of hypertension and hypokalemia are suggestive of high aldosterone levels. But aldosterone and renin levels in AME are low, reflecting a sodium-replete state caused by cortisol-mediated MR activation (182, 183).

The genomic actions of MR action can be divided into early and late phases. Agonist-bound MR acutely (as early as 30 minutes) increases transcription of genes such as the serum-and-glucocorticoid regulated kinase-1 (SGK1) that regulate the activities of the Na+/K+-ATPase and the amiloride-sensitive epithelial sodium channel (ENaC), which participate in distal tubular sodium reabsorption and exert final regulatory control over sodium homeostasis. MR agonism subsequently leads to induction of mRNAs for Na+/K+-ATPase and ENaC subunits (184, 185).

Mice in which the HSD11B2 gene has been inactivated are hypertensive and hypokalemic- a finding that reinforces the enzyme’s physiologic importance (186, 187). Milder genetic or acquired deficiencies in 11β-HSD2 activity can also cause clinical phenotypes. Ingestion of licorice and glycyrrhetinic acid derivatives such as carbenoxolone can cause an AME-like pseudohyperaldosteronism among susceptible individuals by inhibiting 11β-HSD2 (188).

11β-HSD2 is co-expressed with CYP3A isoforms in MR-expressing segments of mammalian renal distal tubules and collecting ducts (80, 84, 126, 189). In addition to glucocorticoids, the two enzymes share some affinity for structurally-related molecules. For instance, bile acid CYP3A substrates and products can competitively inhibit 11β-HSD2 activity (190, 191). In this chapter, I test the hypothesis that 6β-hydroxycorticosterone competitively inhibits 11β-HSD2 and that its effects on sodium transport partly reflect this inhibition.
METHODS

**Cell Culture:** mIMCD-K2 cells, derived from inner medullary collecting ducts of SV40-transgenic male mice (192), were kindly provided by the laboratory of Dr. Bruce Stanton (Dartmouth University) at passage 14. Cells were seeded at a density of 4000 cells/cm\(^2\) and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100,000 U/liter), and streptomycin (100,000 µg/liter); all were purchased from Gibco/Invitrogen. Cells were maintained in 24- or 48-well plates (Costar) in incubators maintained at 37°C and 5% CO\(_2\) until they reached at least 90% confluence.

For gene expression studies and enzyme assays, cells between passages 19 and 22 were grown to 90% confluence maintained in 24- or 48-well tissue culture plates (Costar). The FBS used in these studies was charcoal-dextran-stripped to remove serum steroids, in accordance with published methods (193), by incubating at 45°C for 30 min with an equal volume of 0.25% activated charcoal/0.025% dextran in 0.01 M Tris/HCl (pH 8.0 at 4°C); charcoal was removed by centrifugation.

All steroids and chemicals used for gene expression and enzyme assays were purchased from Sigma-Aldrich, except for 6β-hydroxycorticosterone (Steraloids, Inc.; Newport, RI). Aldosterone, corticosterone, 6β-hydroxycorticosterone, spironolactone, and mifepristone (RU486) were stored as stock solutions (10 mM in 100% ethanol) at -20°C until use. Carbenoxolone was stored in water (10 mM) at 4°C.
Gene Expression Studies: Steroids and other chemicals were diluted to the indicated concentrations in DMEM with 10% fetal bovine serum that was treated with charcoal-dextran to remove steroids. Final ethanol concentrations were 0.1% in all preparations. Suspensions were added to cells for the indicated times, after which RNA was extracted.

TaqMan RT-PCR: Cells in each well were lysed with 200 µL of Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, CA) diluted by half with phosphate-buffered saline (Gibco). RNA was isolated in an RNA purification tray with the ABI Prism 6700 automated nucleic acid workstation (PE Biosystems). RT-PCR was performed in the ABI Prism 7700 sequence detector (PE Biosystems); cDNA was synthesized by reverse transcription prior to PCR in the 7700 system. During amplification, fluorescences of FAM, TAMRA, and ROX (passive reference dyes) were measured by the 7700 sequence detector. For mRNA expression, each sample’s β-actin Cₜ was used as the standard. Relative mRNA levels were calculated with the ddCₜ method: relative expression = 2⁽⁽Cₜ gene of interest – Cₜ β-actin⁾⁾. TaqMan primer and probe sequences are shown in TABLE IV-A.
## TABLE IV-A. List of TaqMan primers and probes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSD11B1</td>
<td>Forward</td>
<td>AGACACAGAAACAGCTATGAAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGATGATCTCCAGGGCGCA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>f-TGACGCCCTAGCTTCTCCCAAAGGAG-q</td>
</tr>
<tr>
<td>HSD11B2</td>
<td>Forward</td>
<td>GACATCAGCCGTGTCTTGGGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGCCAGCGTTGTTAACCAGA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>f-ATCACCAAGGCCCCACACGCGG-q</td>
</tr>
<tr>
<td>MR</td>
<td>Forward</td>
<td>GTGTCAAGCTCTACTTTTACGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACACCCAGAAGCTCCTATCT</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>f-CTGGATCCTCAAGACCTTCCCAAAGATC-q</td>
</tr>
<tr>
<td>GR</td>
<td>Forward</td>
<td>ACATGTAGGTGGGCGGCA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTGTAAGGTTTCTGAATCTGGTA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>f-CCTTGCCCATTCTACGCGCAGAAATC-q</td>
</tr>
<tr>
<td>ENaCα</td>
<td>Forward</td>
<td>AGCGGTCTTTCCAGTGTA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATTGTCTTGCTGACAGT</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>f-CAACACATCCCCAACTGAGGACAGAAATC-q</td>
</tr>
<tr>
<td>ENaCγ</td>
<td>Forward</td>
<td>CACTGGTCGGAAGCGGAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCACAGTCAGAGGTTCATT</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>f-ACAAGGCTTCTAATGTCAAGCCGCAGTTTC-q</td>
</tr>
<tr>
<td>PGK-CYP3A5</td>
<td>Forward</td>
<td>CTTCAGCTGATGATCGACTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TATGGACTGGGCTGCAGCT</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>f-TGGAAAGAAACTGAGTCCACGCGTCTAGTC-q</td>
</tr>
<tr>
<td>CYP3A11</td>
<td>Forward</td>
<td>AGAACTTCTCCTCCAGCGCTTGTA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAGGGAAGCTCATCGCTCCAGTTA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>f-CTAAGGTTTGCACCCACGCTGAGGAGTCAAGT-q</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Forward</td>
<td>AGACATGAGCCTCCACCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGAAGGAACAGTGTGCAGC</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>f-CAAGTCTCTGTGATCAGTCTCCAGGAGC-q</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>Forward</td>
<td>TGCCACCTGGACATCCTCTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACCTGGACACCTGAAAGAAGAC</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>f-TCCCGAGCCGCTGACCCGATGTCAT-G-TAGTq</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward</td>
<td>CTGCCTGACGGCCAGGTGC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAAGAAGGAAGGCTGGAAAGAAAGA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>f-CACTATTGCCAAGCAGCGTCTCCAGGAGG-q</td>
</tr>
</tbody>
</table>

_f, Reporter dye (FAM:6-carboxyfluorescein);_  
_q, Quencher dye (TAMRA: 6-carboxytetramethylrhodamine)_

**Cell Homogenate Enzyme Assays:** Cells were scraped into a homogenization buffer containing 250 mM sucrose and 10 mM Tris-HCl (pH 7.5) with Protease
Inhibitor Cocktail (Sigma). Cells were disrupted by sonication. Homogenates were incubated for 3 h at 37 °C with 10 nM corticosterone and 2 nM [1,2,6,7-\(^3\)H]-B (Sigma; 70-100 Ci per mmol) in homogenization buffer with and without 1 mM NAD. Samples were incubated for 3 h at 37 °C and the reaction was stopped on ice. Steroids were extracted by adding two volumes of ethyl acetate. Samples were vortexed vigorously for 1 min and then centrifuged at 10,000 xg for 5 min. The upper ethyl acetate layer was removed, extraction was repeated, and the extracts were pooled. Ethyl acetate was evaporated from pooled extracts by centrifuging under vacuum pressure. The residue was dissolved in a small amount of ethanol that contained 10 nmol each of unlabeled corticosterone and 11-dehydrocorticosterone. 11\(\beta\)-HSD2 activity was assessed by thin-layer chromatography (TLC) (194). Samples were dotted onto silica gel TLC plates with a 254-nm fluorescent indicator (Whatman). Unlabeled steroid standards were added in separate lanes. Plates were developed in glass beakers containing a mobile phase of 92% chloroform/8% ethanol and dried at room temperature. Plates were visualized under short-wave ultraviolet light; fluorescence-quenched spots aligning with corticosterone and 11-dehydrocorticosterone standards were marked, cut from the plates, and placed separately into vials containing Scintiverse BD Cocktail (Fisher Scientific). After a brief shaking, vials were placed in a LKB RackBeta scintillation counter and counts per minute (CPM) were recorded. The percent conversion for each sample was calculated by dividing the 11-dehydrocorticosterone CPM value by the sum of the corticosterone and 11-dehydrocorticosterone CPM values.
Whole-Cell Enzyme Assays: DMEM/10% charcoal-stripped FBS, containing 2nM [1,2,6,7-3H]-B and indicated concentrations of unlabeled corticosterone, was applied to cells. At the end of incubation, medium from each well was removed for steroid extraction. Sample processing and data collection procedures were conducted as for whole-cell assays. The amount of 11-dehydrocorticosterone produced by each incubation was calculated by multiplying the corresponding percent conversion by the total amount of corticosterone (tritiated and unlabeled) added to the incubation.

Analysis: Substrate-velocity curves were fitted by nonlinear regression with GraphPad Prism (GraphPad Software, Inc.; San Diego, CA). Modeling assumed that conversion of B obeys Michaelis-Menten kinetics and that its inhibition by 6β-OHB was competitive and reversible.

Variation among the means of multiple groups was evaluated with analysis of variance (ANOVA). Pairwise differences were analyzed with the Student’s t-test. Dose- and time-dependent effects were explored by linear regression where noted. Analytical and graphing software included Prism, JMP (SAS Institute, Inc.; Cary, NC), and Microsoft Excel. Unless indicated, all experiments were conducted in triplicate and means and standard deviations are reported.

RESULTS

Gene expression: TaqMan analysis indicated baseline signals corresponding to the mRNAs for MR, GR, HSD11B2, ENaCα, ENaCγ, PPARγ, and endothelin-1 (data not shown). Therefore, it was deemed that mIMCD-K2 cells have an mRNA expression
pattern retain reminiscent of collecting duct cells in vivo. CYP3A11 mRNA was not reliably detected in mIMCD-K2 cells at baseline. HSD11B1 mRNA was also detected and its ddCt value was roughly 7 times that of HSD11B2. The relative signal for GR exceeded that of MR by more than 70-fold.

**Gene expression responses to steroid treatment:** Cells were treated with 10⁻⁶ M corticosterone for up to 8 hours and with varying doses of aldosterone and corticosterone for 24 hours. As shown in FIGURE IV-1, SGK1 mRNA levels respond to corticosterone treatment by 2 hours. ENaCα mRNA was dose-dependently induced by both steroids at 24 hours, although the maximum effect of aldosterone was nearly three times that of corticosterone. The IC₅₀, the dose at which 50% of the maximum effect is produced, was roughly 5x10⁻⁷ M for each.
FIGURE IV-1. mIMCD-K2 cells respond to corticosterone treatment.
A: Treatment with $10^{-6}$ M B from 0 to 8 h produces a short-term increase in SGK1 mRNA response. B: ENaCα mRNA levels are increased aldosterone and corticosterone at 24 h.

Comparison of 6β-hydroxycorticosterone to carbenoxolone: Cells were incubated with varying concentrations of 6β-hydroxycorticosterone and carbenoxolone, the known 11β-HSD2 inhibitor, in the absence of corticosterone for 24 h (FIGURE IV-2A). While some dose effects may be apparent, they were relatively minor. FIGURE IV-2B shows the ability of 6β-hydroxycorticosterone and carbenoxolone to alter the responsiveness of ENaCα mRNA levels to corticosterone. These data demonstrate that 6β-hydroxycorticosterone behaves like carbenoxolone.
in an important manner: its major effects on ENaCα mRNA levels are exhibited only in the presence of corticosterone. 6β-hydroxycorticosterone was generally more effective than carbenoxolone at the same concentrations under most conditions. Most but not all assays indicated a left-shift in the efficacy of corticosterone when 6β-hydroxycorticosterone was added.

**FIGURE IV-2.** 6β-hydroxycorticosterone and carbenoxolone effects on ENaCα mRNA levels.

**A:** Cells were treated for 24 h with varying concentrations of CBX or 6β-OHB alone. **B:** Cells were treated for 24 h with 10^-6 or 10^-5 M of either CBX or 6β-OHB in the presence of B (10^-8 or 10^-7 M).

**6β-hydroxycorticosterone alters ENaCα response to corticosterone:** Data from 24-h incubations of mIMCD-K2 cells with varying concentrations of corticosterone with or without 6β-hydroxycorticosterone, RU486, and/or spironolactone (all at 10^-5 M) may suggest that 6β-hydroxycorticosterone protects the activity of corticosterone. 6β-hydroxycorticosterone may have had some minor effects in this assay on the shape of the dose-effect curve for corticosterone but its major effects occurred when
either MR or GR were blocked. As shown in FIGURE IV-3A, the GR antagonist RU486 (R) produced a two-order left-shift in dose-effect, likely by blocking the low-efficacy GR and thus freeing corticosterone to bind to the high-efficacy MR. But the addition of 6β-hydroxycorticosterone in this setting nearly completely flattens this response, which may reflect 6β-hydroxycorticosterone antagonism of MR, as previously reported (81) and as suggested by FIGURE IV-4. The addition of the MR antagonist spironolactone (S) to corticosterone (FIGURE IV-3B) alone also flattens its effect by a mechanism that is unknown but may involve induction of 11β-HSD2. Adding 6β-hydroxycorticosterone then restores corticosterone’s effect, possibly by inhibiting 11β-HSD2 activity and/or expression (FIGURE IV-7, FIGURE IV-8, and FIGURE IV-9).

FIGURE IV-3. Interaction between GR and MR blockade and 6β-hydroxycorticosterone effects on ENaCα mRNA induction by corticosterone. A: Cells were treated for 24 h with varying concentrations of B and 10^{-5} M RU486 (R), 10^{-5} M 6β-OHB (6b), or both. B: Varying concentrations of B and 10^{-5} M spironolactone (S), 10^{-5} M 6β-OHB, or both.
6β-OHB suppresses ALDO response: Cells were treated with increasing concentrations of aldosterone (ALDO) for 24 hours with and without the addition of $10^{-5}$ M 6β-hydroxycorticosterone. The expected increase of ENaCα mRNA in response to aldosterone was dampened significantly when 6β-hydroxycorticosterone was added (FIGURE IV-4).

![ENaCα mRNA level vs. [ALDO]](chart.png)

**FIGURE IV-4. ENaCα mRNA dose-response with or without $10^{-5}$ M 6β-hydroxycorticosterone.**

mIMCD-K2 cells dose-dependently increase ENaCα mRNA levels at 24 h in response to ALDO treatment, but $10^{-5}$ M 6β-OHB significantly blunts this response.

mIMCD-K2 cells convert corticosterone: Cells were incubated with 2 nM [$^3$H]-corticosterone and 100 nM unlabeled corticosterone for the time-points indicated in FIGURE IV-5A, which shows a time-dependent increase in corticosterone conversion. A decrease in the apparent ratio of 11-dehydrocorticosterone to corticosterone at longer time-points (not shown) may represent the action of 11β-HSD1. Therefore, 30-min incubations were performed for substrate-velocity and
inhibition experiments. In order to establish that corticosterone conversion reflected 11\(\beta\)-HSD2 activity, cell homogenates were assayed with and without the addition of 1mM NAD\(^+\) (an 11\(\beta\)-HSD2 cofactor) and 100 nM Ca\(^{2+}\) (an 11\(\beta\)-HSD2 inhibitor), as shown in FIGURE IV-5B. Conversion was stimulated by NAD\(^+\) and inhibited by Ca\(^{2+}\), which is consistent with 11\(\beta\)-HSD2 activity (195).

**FIGURE IV-5. Corticosterone conversion by mIMCD-K2 cells.**
A. Conversion is time-dependent. Separate assays showed an apparent decrease in the ratio of 11-dhB to B at longer time points (90-120 min). B. 1-hr B conversion is stimulated by NAD\(^+\) and inhibited by Ca\(^{2+}\), consistent with 11\(\beta\)-HSD2 activity.

**11\(\beta\)-HSD2 kinetics:** Data from 30-min incubations of cells with 2 nM \[^3\text{H}\]-corticosterone and varying unlabeled corticosterone concentrations were fitted by nonlinear regression, assuming Michaelis-Menten kinetics. The substrate-velocity curve is shown in FIGURE IV-6. The estimated Michaelis-Menten constant (\(K_m\)) for these data is roughly 150 nM (SE= 35 nM).
FIGURE IV-6. Whole-cell 11β-HSD2 substrate-velocity curve. Cells were incubated for 30 min with 2 nM $[^3]$H-B and varying concentrations of unlabeled B.

**6β-hydroxycorticosterone inhibits corticosterone conversion:** Cells were incubated for 30 min with 2 nM $[^3]$H-corticosterone and indicated corticosterone and 6β-hydroxycorticosterone concentrations to test the hypothesis that 6β-hydroxycorticosterone competitively inhibits 11β-HSD2 activity (FIGURE IV-7A). Substrate-velocity and double-reciprocal plots (inset) are shown. The best-fit estimate for the inhibitory constant ($K_i$) for these data is 4.7 nM (SE= 2.3). The relative abilities of corticosterone and 6β-hydroxycorticosterone to inhibit the 30-min conversion of 2 nM $[^3]$H-B was also examined (FIGURE IV-7B). At all concentrations, 6β-hydroxycorticosterone produced greater inhibition than did corticosterone.
FIGURE IV-7. 6β-hydroxycorticosterone inhibits 11β-HSD2 activity. Cells were incubated for 30 min with 2 nM [3H]-B and with the indicated concentrations of unlabeled B or of 6β-OHB. A. Substrate-inhibitor profile. Estimated $K_i=4.7$ nM. B. B and 6β-OHB dose-dependently inhibit [3H]-B conversion.

6β-hydroxycorticosterone suppresses 11β–HSD2 mRNA and activity: Cells were cultured in $10^{-7}$ M corticosterone +/- $10^{-5}$ M 6β-hydroxycorticosterone. By 2 h, corticosterone had suppressed by half the mean 11β–HSD2 mRNA level (FIGURE IV-8A), which recovered from 2-8 h in the absence of 6β-hydroxycorticosterone; in
its presence, recovery was flat. In separate studies, the time- and dose-dependence of this effect were examined for 11β–HSD1 and 2 (FIGURE IV-8B and C). With corticosterone alone, HSD1 mRNA levels decreased from 4 to 8 h while 11β–HSD2 levels increased. 6β-hydroxycorticosterone dose-effect trends on 11β–HSD1 at 2 and 4 h were marginal in significance (P=0.04 and 0.09, respectively). At 8h, only the trend for 11β–HSD2 was significant (P=0.001).

FIGURE IV-8. 6β-hydroxycorticosterone affects 11β-HSD mRNA levels. Cells were incubated up to 8h with 10⁻⁷ M B and the indicated concentrations of 6β-OHB. A. 6β-OHB reduces 11β-HSD2 mRNA recovery after suppression by B. B and C. 6β-OHB dose-dependently inhibit [³H]-B conversion; 6β-OHB was more potent than B at all doses.
Culturing mIMCD-K2 cells for 24 h in $10^{-7}$ M corticosterone alone or with the 11β–HSD2 inhibitor carbenoxolone ($10^{-5}$ M) or 6β-hydroxycorticosterone ($10^{-6}$ or $10^{-5}$ M) suppressed 11β–HSD2 activity (FIGURE IV-9).

**FIGURE IV-9.** 6β-hydroxycorticosterone suppresses 11β-HSD2 activity. Cells were incubated for 24 h with $10^{-7}$ M B and the indicated concentrations of CBX and 6β-OHB. $10^{-5}$ M 6β-OHB suppressed 11β-HSD2 activity.

**DISCUSSION**

The primary purpose of these experiments was to determine whether 6β-hydroxycorticosterone, a product of CYP3A-catalyzed metabolism, alters renal sodium transport by changing the expression of ENaC subunits. The secondary purpose was to identify potential mechanisms and to specifically test the hypothesis that 6β-hydroxycorticosterone inhibits the 11β-HSD2 enzyme. Cultured mouse inner medullary collecting duct cells have been used previously as *in vitro* models of steroid regulation of sodium transport.
The major findings of these experiments are that 6β-hydroxycorticosterone competitively inhibits 11β-HSD2 activity and that it has an unanticipated effect upon 11β-HSD2 expression. 6β-hydroxycorticosterone inhibition of 11β-HSD2 activity appears to occur in a physiologically compatible range. Results from whole-cell enzyme assays indicate that corticosterone conversion is consistent with Michaelis-Menten kinetics. The estimated $K_m$, roughly 150 nM, is higher than published $K_m$ values for 11β-HSD2, which range from 10-100 nM. It is not clear whether this difference is meaningful, but the presence of 11β-HSD1 in these cells may be responsible for a higher apparent $K_m$ due to back-conversion of product to substrate. The derived $K_i$ for 6β-hydroxycorticosterone in this reaction is around 5 nM.

Data regarding the intracellular levels of 6β-hydroxyglucocorticoids are not available from the literature. Human plasma 6β-hydrocortisol levels average 1-3 nM but are higher in hypercortisolemic states, in which they may exceed 10 nM, and in tissues in which 6β-hydrocortisol is produced, such as the adrenal gland (129, 149). The levels of 6β-hydrocortisol in the urine can reach the micromolar range, which may indicate high intrarenal concentrations. It is very likely that the ratio of 6β-hydrocortisol to cortisol in renal epithelial cells exceeds that in plasma. The efflux transporter P-glycoprotein (P-gp), encoded by the $MDR1$ gene, limits the intracellular concentrations of its steroid ligands, including glucocorticoids (117, 196-198). Steroid transport by P-gp is sensitive to structural and physicochemical properties of these compounds. Introduction of a 6β-hydroxyl group to cortisol greatly reduces transport by LLC-PK porcine kidney cells with or without transfection with MDR1 (199). The in vivo relevance of this finding would be that the intracellular ratio of 6β-
hydroxycortisol to cortisol may be higher than that seen in plasma, as cortisol is pumped out of the cell and 6β-hydroxycortisol formed intracellularly by CYP3A is cleared less efficiently.

CYP3A11 expression was not reliably seen in these cells. CYP3A11 mRNA is detectable in adult mouse kidney, although at relatively low levels compared to CYPs from families 1, 2, and 4 (200). CYP3A5 expression in medullary collecting ducts has been confirmed in humans (84). In future studies, it will be worth asking what the effects of actual CYP3A expression would be in these cells.

The higher relative mRNA levels for GR relative to those for MR in these cells parallel a GR predominance previously noted in cultured collecting duct cells (126). The higher relative mRNA levels for 11β-HSD1 relative to those for 11β-HSD2 are consistent with results from other investigators showing 11β-HSD1 in rat distal tubules (201) and higher TaqMan and Western blot signals for 11β-HSD1 than for 11β-HSD2 in the inner medulla (202).

The in vivo relevance of these findings is unclear. Inhibition of 11β-HSD2 should in theory have an antinatriuretic effect. But the apparent ability of 6β-OHB to antagonize the MR and the possible dependence of 6β-hydroxycorticosterone effects on MR or GR blockade makes the proposed role of 6β-hydroxycorticosterone in normal renal and systemic physiology somewhat difficult to predict. Further in vitro and in vivo studies should detail more finely define the dose effects of 6β-hydroxycorticosterone and explore its effects on not merely ENaC mRNA but also levels and activities of other effectors of sodium balance, such as Na⁺/K⁺-ATPase, Na⁺/K⁺/Cl⁻ cotransporter (NKCC), and renin-angiotensin-aldosterone system
components. But whether or not 6β-OHB proves to be a major agent in human physiology and health, studies of its effects should provide new insights into the biology of renal epithelia.
CHAPTER V: IN VIVO MODELS OF CYP3A PHYSIOLOGY
SUMMARY

Animal and human data suggest that high CYP3A activity in the kidney may cause increased renal sodium transport. Cytochrome P450 3A enzymes have been proposed to alter renal sodium reabsorption by catalyzing the conversion of glucocorticoids (cortisol or corticosterone) to their 6β-hydroxylated metabolites with altered activity. The mechanisms by which these metabolites act in the kidney have not previously been identified. Excessive reabsorption of sodium by the kidney may cause increased plasma volume and a resultant hypertension. Previous studies of a possible relationship between CYP3A activity and blood pressure and/or renal function have been mostly associative, without establishing clear causation. These studies are an attempt to establish whether increased 6β-hydroxycorticosterone levels and or increased expression of CYP3A enzymes in mice can increase sodium sensitivity and/or blood pressure.

INTRODUCTION

The search for genetic causes of common diseases such as hypertension is difficult because disease-causing genetic variants are generally either rare or have little effect individually. It has been suggested that the cytochrome P450 3A (CYP3A) enzyme system could have large effects on human health. Among adults, CYP3A4 and CYP3A5 exhibit variable expression which is partly controlled by genetic variance. CYP3A4 is variably but universally expressed primarily in the liver and intestines; CYP3A5 is found in the gastrointestinal system and in the renal epithelia
of some individuals who constitute a minority among some populations and a majority among others. The large active sites of CYP3A enzymes allow them to catalyze the oxidative metabolism of a wide variety of endogenous and exogenous chemicals, including most therapeutic drugs.

The work of Watlington and colleagues implicated CYP3A enzymes in the control of renal sodium balance and blood pressure. CYP3A content and catalytic activity in the kidneys of spontaneously hypertensive rats (SHR) are higher than in normotensive Wistar-Kyoto rats (80, 85) and treatment of SHR with the CYP3A inhibitor troleandomycin lowers BP (85). Several human diseases associated with elevated BP, including Cushing’s syndrome (129) and pre-eclampsia (80), are characterized by increased urinary excretion of 6β-hydroxycortisol, a marker of in vivo CYP3A activity (85, 130). Plasma levels of 6β-hydroxycortisol have been reported to be elevated in essential hypertensives (87).

A role for CYP3A enzymes in blood pressure control appears plausible. CYP3A-mediated 6β-hydroxylation is a metabolic pathway, although a minor one, for steroids such as the glucocorticoid corticosterone. 6β-hydroxycorticosterone stimulates sodium transport across A6 toad kidney cells and sodium retention in rats (80-82). Previous evidence indicates that 6β-hydroxycorticosterone modulation of A6 cell sodium transport by acting through an unidentified binding site (80, 81). Immunohistochemistry demonstrates CYP3A proteins in rat (80, 131) and human kidney (84), including the distal tubules and collecting ducts, the principle sites at which aldosterone stimulates sodium reabsorption. Similar to that of 11β-
hydroxysteroid dehydrogenase type 2, renal CYP3A activity has been proposed to regulate glucocorticoid occupancy of the mineralocorticoid receptor (MR) (83).

In addition, work by a number of investigators has indicated possible associations between CYP3A expression and blood pressure or sodium balance in some human studies. In order to move beyond association and establish causation, it will likely be necessary to employ animal models so that the effects of modulating CYP3A activity upon in vivo physiology can be measured.

METHODS

6β-hydroxycorticosterone injection: Wild-type C57BL/6 male mice were separated into two groups (n=4 each) that received a daily intraperitoneal injection of either 6β-hydroxycorticosterone (1 µg/g body weight) in PBS or with PBS alone. Each group received a normal-chow diet and drinking water ad libitum. Blood pressures were measured daily for 6 days. Drinking water was then replaced with 0.9% saline, injections were continued, and blood pressure measurements were repeated for an additional 6 days.

PGK-CYP3A5 tg mice: Functional human CYP3A5 cDNA in a pENTR/SD/DTOPO cloning vector (Invitrogen) was kindly provided by the laboratory of Erin Schuetz (St. Jude’s Children’s Research Hospital, Memphis, TN). CYP3A5 cDNA was excised from the pENTR vector and ligated into a transgenic vector directed by a phosphoglycerate kinase (PGK) promoter (UNC Animal Models Core). The vector was linearized and microinjected into male pro-nuclei of fertilized C57BL/6 oocytes.
by standard techniques. Founders were identified by PCR of tail or toe DNA using primers that anneal within the PGK promoter (AGCAGTGACTCTGGGGGCAG; forward) and CYP3A5 cDNA (GGAGTTGACCTTCATACGTTCCCCAC; reverse). Founders were mated with wild-type C57BL/6 in order to produce transgenic and wild-type offspring.

**ApoAI-ApoCIII-CYP3A5 mice:** Human CYP3A5 cDNA was ligated into a targeting construct containing mouse apoA1 gene and a 3’ fragment of the apoC3 gene (203). Inserting a transgene into this locus produces high expression of the transgene in the liver and intestines (204). Chimeric males were mated with wild-type C57BL/6 females. Transgenic and wild-type agouti F1 offspring were used for experiments. Genotyping of ApoCYP3A5 mice was performed by PCR amplification of tail or toe DNA using oligonucleotide primers that anneal within the PGK promoter and CYP3A5 cDNA, as for PGK-CYP3A5 mice.

**Blood Pressure Measurement:** Noninvasive computerized tail cuff sphygmomanometry was used for BP measurements of conscious mice. Each trial consisted of six consecutive days of measurements. On each day, a series of 10 inflations is performed to allow the mouse to acclimatize to the apparatus, after which 30 inflations and measurements were performed. The average of the six daily means was used to represent the mean blood pressure of each animal and is used for statistical analysis.
Diets: PGK-CYP3A5 were fed either normal chow, a high-salt diet (8% sodium), and a low-salt diet (1% sodium). ApoCYP3A5-transgenic and wild-type mice were crossed over between normal chow, a high-salt diet (8% sodium), and a high-fat diet (42% of calories from fat). All diets were from Harlan-Teklad. Mice were allowed to acclimate to each diet for one week prior to each 6-day round of blood pressure measurement.

RESULTS

6β-hydroxycorticosterone injection did not influence sodium sensitivity:
Ingestion of saline non-significantly increased systolic and diastolic blood pressure and lowered heart rate among 6β-hydroxycorticosterone- and PBS-treated mice. Injection with 6β-hydroxycorticosterone as opposed to PBS did not significantly affect these responses. Also, 6β-hydroxycorticosterone did not exhibit a BP-raising effect and nonsignificantly lowered SBP and raised HR (FIGURE V-1).
FIGURE V-1. 6β-hydroxycorticosterone affects blood pressure and heart rate.

**Effects of CYP3A5 gene introduction on blood pressure:** PGK-CYP3A5 transgenic demonstrated expression of CYP3A5 mRNA in the liver and kidney and suppression of endogenous mouse CYP3A11 in these same tissues (FIGURE V-2). Renal CYP3A11 expression was 1000-fold lower than hepatic expression for both wild-type and transgenic mice. These mice showed trends towards higher systolic (SBP) and diastolic blood pressures (DBP) on low- (LS) and high-salt (HS) diets. Mean SBP and DBP were nearly equal between transgenic and wild-type mice on a normal-salt (NS) diet (FIGURE V-3). Among the ApoAI/CIII-CYP3A5 mice there was no apparent genotype effect on blood pressure but there was a diet effect among
wild-type and CYP3A5-positive mice such that high-fat diet lowered blood pressure (FIGURE V-4).

FIGURE V-2. Relative hepatic and renal expression of CYP3A11 mRNA in wild-type and PGK-CYP3A5 transgenic mice.

FIGURE V-3. Diet and PGK-CYP3A5 genotype effects on systolic and diastolic blood pressure.
FIGURE V-4. Dietary effects on systolic blood pressure and heart rate in ApoAI/CII-CYP3A5 transgenic and wild-type mice.
Mice were fed normal chow (NC), high-salt (HS), or high-fat (HF) diet. Bars show means and standard deviations of systolic blood pressure (SBP) and heart rate (HR).

DISCUSSION

The multiple CYP3A genes in humans, mice, and other animals reflect species-specific gene duplication and recombination events. There is thus no clear mouse ortholog of the human CYP3A5 gene, although the mouse and human CYP3A genes are obviously homologous. The attempt to create a mouse model that perfectly replicates the in vivo distribution and activity of human CYP3A5 is difficult in theory. In this study, it was attempted to circumvent the potential problems of achieving the proper tissue-specific distributions of "CYP3A5-like" activity by employing multiple approaches to model the potential hypertensive effects of CYP3A and/or 6β-hydroxycorticosterone activity.

CYP3A7 transgenic mice with a metallothionein promoter driving zinc-induced hepatic expression have previously been made (205, 206). These animals show increased serum testosterone levels, higher liver, kidney, and uterine weights, and
decreased testis weight. The reasons for these effects were not studied but testicular expression of CYP3A7 was confirmed. The authors speculated that the higher testosterone in conjunction with smaller testes may have reflected transgene disruption of a gene responsible for testosterone metabolism, such as the gene encoding steroid 5α-reductase. It is more likely, however, that products of testicular CYP3A-catalyzed reactions, such as 6β-hydroxytestosterone, alter activity or expression of 5α-reductase or a related enzyme. Physiologic parameters, such as blood pressure, have not been reported for other humanized CYP3A models as they have for the models described in this chapter.

Repression of endogenous mouse CYP3A mRNA was not seen in the CYP3A7-transgenic model (207). But a 50% reduction of mouse hepatic CYP3A protein levels was observed in female mice bearing a CYP3A4 transgene driven by the full CYP3A4 promoter; transgenic males showed an age-dependent increase in murine hepatic CYP3A protein levels that exceeded those of wild-type males by 12 weeks of age (208). Mechanisms underlying these effects and differences between models have not been explained.

The possible effects of 6β-hydroxycorticosterone on blood pressure and heart rate at baseline and in response to dietary sodium modification may be further investigated in intact and adrenalectomized mice. Doses of 6β-hydroxycorticosterone may be varied in the presence or absence of 11β-HSD2 inhibition (with carbenoxolone or glycyrrhetinic acid), and antagonism of the mineralocorticoid or glucocorticoid receptors (with spironolactone or RU486,
respectively). It will be informative to measure not only hemodynamic parameters but also indicators of renal function and sodium balance.

It is similarly necessary to investigate the possible effects of PGK-CYP3A5 transgenesis on hemodynamics and renal function. It will be informative to compare the magnitude and distribution of CYP3A expression and activity between wild-type and transgenic animals.
CHAPTER VI: SYSTEMATIC REVIEW OF STUDIES OF THE ASSOCIATION OF 
CYP3A5 GENOTYPE WITH BLOOD PRESSURE AND HYPERTENSION
SUMMARY

Isoforms of the human CYP3A subfamily are well known as hepatic drug-metabolizing enzymes. Beyond their pharmacologic importance, CYP3A enzymes have established and hypothesized roles in endogenous physiologic processes. In animal and in vitro models, 6β-hydroxycorticosterone, a product of CYP3A enzyme activity, increases renal sodium transport and is associated with increased blood pressure. Among humans, increased plasma levels and urinary excretion of 6β-hydroxycortisol have been seen in diseases associated with increased blood pressure. Polymorphic human renal CYP3A activity primarily reflects the contributions of CYP3A5, the expression of which is primarily controlled by known single nucleotide polymorphisms. These observations led to the hypothesis that expression-predicting CYP3A5 genotypes could predict renal sodium transport and blood pressure. Studies have examined associations between the CYP3A5 A6986G SNP and blood pressure, hypertension, renal function, and other metabolic phenotypes among American and African blacks, American and European whites, and various Asian groups. A review of studies to date shows that results have been quite variable. Summary measures show very minor and insignificant associations of CYP3A5 genotype with BP and HTN among whites, possible associations among Asians, and variable results among blacks. These observations support the notion that development of robust, conventional molecular epidemiologic case-control studies to address these questions, including gene-gene and gene-environment interactions, will be timely.
INTRODUCTION

The cytochrome P450 (CYP) enzyme superfamily is an exceptionally diverse group divided into roughly 237 numbered families on the basis of sequence homology (1, 2). CYP1-3 families comprise the phase I drug-metabolizing enzymes (4), which catalyze substrate functionalization via reactions such as hydroxylation and demethylation that increase aqueous solubility or introduce attachment sites for bulkier moieties added by phase II enzymes. CYP proteins are expressed in the liver and throughout the gastrointestinal tract, where the bulk of first-pass drug metabolism occurs (5).

The human CYP3A enzymes- i.e., the members of family 3, subfamily A of the CYP superfamily- are numbered 4, 5, 7, and 43. The CYP3A isoforms have extensively overlapping substrate specificities. Among the human CYP subfamilies, CYP3A is the most prominent. Its members are the most highly expressed in the liver and intestines and they catalyze metabolism of a tremendous variety of substrates, including half or more of currently marketed drugs (9). The bulk of adult human CYP3A activity results from the contributions of the CYP3A4 and CYP3A5 enzymes.

Gastrointestinal CYP3A4 activity is variable but universal (209, 210). But CYP3A5 expression is clearly polymorphic (25, 42). Among individuals who express CYP3A5, it is the primary component of extrahepatic CYP3A activity. While CYP3A4 and 5 may be co-expressed in extra-gastrointestinal sites such as the placenta (27), CYP3A5 is also found in sites from which CYP3A4 absent- i.e., the kidney (28-30), including proximal and distal tubules and collecting ducts (21, 31); adrenal zona
glomerulosa (21, 31); bronchial and alveolar respiratory epithelia (32); and anterior pituitary gland (33).

The human CYP3A5 gene is located on band q22.1 of chromosome 7. The CYP3A5 gene encompasses roughly 31.8 kbp and has 13 exons. The CYP3A5 promoter contains a basal transcription element (–35 to –50), binding sites for AP-3 and p53. The CYP3A5 5'UTR displays response elements for the pregnane X receptor (PXR), the main effector of CYP3A transcriptional induction (211). Also found in the CYP3A5 promoter are glucocorticoid and estrogen response elements and hepatocyte nuclear factor-4 and -5 binding sites (212). The CYP3A5 holoprotein consists of 502 amino acids with a molecular weight of 57.1 kDa.

In addition to their roles in the metabolism of drugs and other exogenous chemicals, CYP3A enzymes also constitute a significant metabolic pathway for lipophilic endogenous molecules, as reviewed in Chapter I. The risk for diseases and conditions such as breast and prostate cancer and early puberty (153, 154, 157, 161) have been hypothesized to be modulated by CYP3A-mediated transformation of estrogens and androgens. Most relevant to this review is the specific status of CYP3A enzymes, and especially CYP3A5, as glucocorticoid 6β-hydroxylases.

CYP3A5 is an attractive candidate as a gene influencing hypertension. Hydroxysteroid products of CYP3A-catalyzed reactions modulate sodium transport across cultured toad kidney cells. CYP3A enzymes are expressed in rodent and human renal tubules, including the distal tubules and collecting ducts, the major sites at which mineralocorticoids control renal sodium reabsorption. CYP3A enzymes are overexpressed in the kidneys of the spontaneously hypertensive rat (SHR) relative
to the related normotensive Wistar-Kyoto strain, and treatment of SHR with a CYP3A inhibitor reduces blood pressure. Injection of SHR with 6β-hydroxycorticosterone leads to sodium retention. Excess urinary 6β-hydroxycortisol excretion has been noted in diseases marked by increased blood pressure, such as Cushing’s syndrome and pre-eclampsia. The response of urinary 6β-hydroxycortisol excretion to sodium loading has been reported to differ between individuals whose blood pressure is sodium-sensitive and those who are sodium-resistant. A study of essential hypertensives revealed higher plasma 6β-hydroxycortisol levels among hypertensives than among normotensives. The observation that the probability of CYP3A5 expression followed a roughly equatorial-to-polar gradient suggested the possibility that the evolutionary importance of renal CYP3A5 activity may have been an ability to prevent dehydration by enhancing sodium resorption. This hypothetical antinatriuretic effect was proposed to lead to increased blood pressure in situations in which sodium availability exceeds requirements, as in the modern Western diet. Perhaps fittingly, CYP3A5 expression is most frequently observed among African-Americans and Black Africans, among whom global rates of hypertension and sodium sensitivity are the highest (123, 124). The possibility that CYP3A5 was a “hypertension gene” was also intriguing because of CYP3A5’s activity as a drug metabolizing enzyme, thus presenting CYP3A5 as a possible “double effector” that could control both the likelihood of developing hypertension and the likelihood of responding to antihypertensive treatment.

The 2001 report by Kuehl et al. (25) of the major genetic variant (A6986G) underlying polymorphic CYP3A5 expression enabled the development of studies
associating CYP3A5 expression with a variety of pharmacologic and physiologic phenotypes. Thus, Givens et al. (30) examined the association of the CYP3A5 A6986G SNP with blood pressure and other clinical findings among a small group of young, mostly college-aged, healthy African-Americans who had been screened for a pharmacogenetic study. Significant associations with CYP3A5 genotype were noted for systolic blood pressure and creatinine clearance. Subsequent studies by others investigating CYP3A5 genotype association with blood pressure and/or the diagnosis of hypertension have produced variable results. This review is an attempt to quantitatively and qualitatively review the current data and assess the state of the evidence. Significant variability exists among studies in terms of subject selection, methodology, and results. Future analyses of this type require more standardized reporting of subject characteristics.

**METHODS**

ISI Web of Science, Medline, CINAHL, and EMBASE were searched for all articles and abstracts mentioning both CYP3A5 and blood pressure or both CYP3A5 and hypertension. Materials that did not include an association of CYP3A5 genotype and blood pressure or hypertension as a primary or secondary analysis were removed.

**Statistical analyses:** For studies included in the meta-analysis, the main outcome measures extracted were systolic blood pressure and the proportion of hypertensives among CYP3A5 expressers and nonexpressers. For the purpose of pooled analyses, statistics that could be used to estimate the variances of the
outcome measures were also recorded. These included standard deviations or standard errors, or if these statistics were not given, confidence intervals or exact t or P values. For studies of systolic blood pressure, the mean difference between the CYP3A5 expresser and nonexpresser groups and the pooled standard deviation were used to calculate a z score. For hypertension, z scores were calculated from odds ratio of hypertension among expressers versus nonexpressers. It was assumed that genotype effects were not different between males and females, so data from studies reporting data for males and females separately were transformed by standard methods. Mean effect sizes were calculated by weighting each trial by the inverse of the variance. Funnel plot asymmetry was used to detect publication biases among the studies.

RESULTS

Fifteen publications were identified comprising 10,859 subjects. Relevant summary data, including means and measures of variation, were extracted from each study. The list of identified studies is displayed in TABLE VI-A. For meta-analysis, studies that examined healthy subjects were included. Data from suitable studies were entered into MIX (Meta-analysis with Interactive Explanations), a freely-available and user-friendly software (213). MIX was used for all analyses.

A random effects model was used to take into account inter-study heterogeneity. Publication bias was unapparent on the basis of funnel plots. Forest plots and summary measures for systolic blood pressure and hypertension among all subjects, healthy Whites, healthy Blacks, and healthy Asians were generated.
The summary estimate for \textit{CYP3A5} genotype effect among all subjects was a 0.29-mm Hg increase (P = 0.41) in systolic blood pressure among \textit{CYP3A5\textasciitilde1} non-carriers (FIGURE VI-1). Sizable or significant effects of genotype upon systolic blood pressure or hypertension among any group were unapparent among all subgroups.

![Forest plot of studies associating systolic blood pressure with \textit{CYP3A5} genotype.](image)

**DISCUSSION**

The absence of an apparent effect of \textit{CYP3A5} genotype on blood pressure or hypertension may indicate that in reality no true effect exists, or that any actual effect is swamped by other genetic and environmental variables, or that studies have been too varied in methodology to allow for adequate statistical summation. The results of
this meta-analysis might be considered alongside those of a recent meta-analysis of genetic association studies of the M235T angiotensinogen polymorphism. Despite a clear role for angiotensinogen in the control of BP and the ability of M235T to predict angiotensinogen plasma levels, meta-analysis demonstrates unconvincing evidence for M235T as a predictor of blood pressure or the risk of hypertension (214).

While more than 10,000 subjects have been included in published CYP3A5-blood pressure association studies, only about 800 of these subjects are classified as black or African-American, African, or of African descent. For various reasons, this figure may represent a significant underrepresentation of the subjects most likely to exhibit a CYP3A5 effect. Rigorous study of a role for CYP3A5 in blood pressure and related parameters is likely to require greater recruitment of black subjects.

Because of heterogeneity among studies, this analysis is presented only as an assessment of the state of the evidence, not as a definitive conclusion about the effects of CYP3A5 genotype. The results support the notion that development of robust, conventional molecular epidemiologic case-control studies to address these questions, including gene-gene and gene-environment interactions, will be helpful.
# TABLE VI-A. Studies of CYP3A5 genotype and blood pressure or hypertension

<table>
<thead>
<tr>
<th>Study</th>
<th>Population(s)</th>
<th>N</th>
<th>Major Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Givens et al. 2003 (30)</td>
<td>AA</td>
<td>9</td>
<td>*1 allele associates with higher SBP, PP, CrCl.</td>
</tr>
<tr>
<td>Kreutz et al. 2005 (169)</td>
<td>CA (Dutch)</td>
<td>43</td>
<td>*1 allele associates with lower SBP, PP, and UNa among women.</td>
</tr>
<tr>
<td>Fromm et al. (164)</td>
<td>CA (German)</td>
<td>0</td>
<td>*1/*3 genotype associates with lower SBP, higher serum ALDO.</td>
</tr>
<tr>
<td>Ho et al. 2005 (165)</td>
<td>CA</td>
<td>0</td>
<td>*1 allele associates with HTN among AA.</td>
</tr>
<tr>
<td></td>
<td>Study 1</td>
<td>27</td>
<td>*1 allele associates with higher insulin resistance among CA.</td>
</tr>
<tr>
<td></td>
<td>Study 2</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Kreutz et al. 2004 (170)</td>
<td>Renal Tx:</td>
<td>6</td>
<td>No association of *1 allele with BP.</td>
</tr>
<tr>
<td></td>
<td>Recipient Donors</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Kivisto et al. 2005 (168)</td>
<td>CA (Finnish,</td>
<td>0</td>
<td>*1 allele associates with HTN; MDR1 3435C&gt;T does not.</td>
</tr>
<tr>
<td></td>
<td>age ≥75)</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Bochud et al. 2006, 2007 (54, 162)</td>
<td>East African</td>
<td>67</td>
<td>*1 allele predicted age-dependent increase in daytime SBP, DBP. Renal Na reabsorption decreased by age in nonexpressers but in expressers.</td>
</tr>
<tr>
<td>Yin et al. 2006</td>
<td>Chinese</td>
<td>7</td>
<td>Nonsignificant associations between 6986 genotype and SBP, DBP, HR, and CrCL.</td>
</tr>
<tr>
<td>Lieb et al. 2004 (172)</td>
<td>CA (German)</td>
<td>5</td>
<td>No genotype association with BP, left ventricular mass or geometry. Mild association with eGFR.</td>
</tr>
<tr>
<td>Neville et al. 2005;</td>
<td>Nigerian males</td>
<td>35</td>
<td>No genotype association with SBP, DBP, BMI.</td>
</tr>
<tr>
<td>Marunde et al. 2006</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Population</td>
<td>Numbers</td>
<td>Comparison/Result</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------------------</td>
<td>---------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Jin et al. 2008 (214)</td>
<td>Japanese males</td>
<td>0 8 8 16</td>
<td>7-mm Hg-higher SBP among *1/*3 vs. *3/*3</td>
</tr>
<tr>
<td>Suh et al. 2006 (215)</td>
<td>Korean cardiac patients</td>
<td>33 122 193 348</td>
<td>No significant differences in rates of HTN or DM.</td>
</tr>
<tr>
<td>Kim et al. 2006 (216)</td>
<td>Korean males</td>
<td>2 16 24 40</td>
<td>No association of *1 with SBP, DBP, or HR.</td>
</tr>
</tbody>
</table>
REFERENCES


4. Williams ET, Rodin AS, Strobel HW. Defining relationships between the known members of the cytochrome P450 3A subfamily, including five putative chimpanzee members. Mol Phylogenet Evol 2004; 33:300-308.


29. Lasker JM, Chen WB, Wolf I, Bloswick BP, Wilson PD, Powell PK. Formation of 20-hydroxyeicosatetraenoic acid, a vasoactive and natriuretic eicosanoid,


58. Fukuen S, Fukuda T, Maune H, Ikenaga Y, Yamamoto I, Inaba T, Azuma J. Novel detection assay by PCR-RFLP and frequency of the CYP3A5 SNPs,


135. Goto M, Masuda S, Kiuchi T, Ogura Y, Oike F, Okuda M, Tanaka K, Inui K. CYP3A5*1-carrying graft liver reduces the concentration/oral dose ratio of


143. Lin HL, Hollenberg PF. The inactivation of cytochrome P450 3A5 by 17alpha-ethynylestradiol is cytochrome b5-dependent: metabolic activation of the ethynyl moiety leads to the formation of glutathione conjugates, a heme adduct, and covalent binding to the apoprotein. *J Pharmacol Exp Ther* 2007; 321:276-287.


