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Expression And Regulation Of Human Cytochrome P450 4F Isoforms In Tissue Samples And Under Tnf-Alpha Challenges

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A

DISSERTATION

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By

Jordan Craig Bell, B.S.

Houston, Texas,

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Dedication

This work is dedicated to my parents, Charlean J. Harris and Ivy Bell Sr.

Acknowledgments

I want to express my gratitude to Dr. Henry W. Strobel for offering me this great opportunity to do my dissertation research in his laboratory. Dr. Strobel has been a great mentor and support system throughout this process. I have learned many new techniques and received the opportunity to attend many conferences to expand my scientific knowledge. I also want to thank my supervisory committee for their helping hand in providing structure to my project and helping to make it a novel and great research project. I extend my thanks to all the previous members of the lab, Auinash, Cheri, Dan, Eric, Jade, Lepa, Sayee and Ying for their friendship and help. I also want to thank Dr. Dollett White with the Department of Pathology at the University of Texas Health Science Center at Houston for participating in a collaboration that provided Human tissue samples for my research project. I also want to thank the labs of Dr. Jianping Jin, Dr. Eric Wagner, Dr. Jaqueline Hecht, and Dr. Michael Gambello for their assistance in helping me learn new techniques that I needed to finish my research project.

I want to give special thanks to my family for being around when I needed an escape from research, and for just being there when I needed them. I want to express my love and appreciation for all of you and let you know I couldn't have done this without you.

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Expression and Regulation of Human Cytochrome P450 4F Isoforms in Tissue Samples and Under TNF-*α* Challenges

Publication No._____

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CYP4F (Cytochrome P4504F) enzymes metabolize endogenous molecules including leukotrienes, prostaglandins and arachidonic acid. The involvement of these endogenous compounds in inflammation has led to the hypothesis that changes in the inflamed tissue environment may affect the expression of CYP4Fs during the pro-inflammatory state, which in turn may modulate inflammatory conditions during the anti-inflammatory state. We demonstrated that inflamed tissues have different levels of CYP4F isoform expression profiles in a number of human samples when compared to the average population. The CYP4F isoform expression levels change with the degree of inflammation present in tissue. Further investigation in cell culture studies revealed that inflammatory cytokines, in particular TNF-α, play a role in regulating the expression of the CYP4F family. One of the isoforms, CYP4F11, had different characteristics than that of the other five CYP4F family members. CYP4F11 metabolizes xenobiotics while the other isoforms metabolize endogenous compounds with higher affinity. CYP4F11 also was expressed at high quantities in the brain, and was up-regulated by TNF-α, while the other isoforms were not expressed at high quantities in the brain and were down-regulated by TNF-α. We identified the AP-1 protein of the JNK pathway as the signaling protein that causes significant increase in CYP4F11 expression.

Since TNF-α stimulation causes a simultaneous activation of both JNK pathway and NF-κB signaling, we investigated further the role that NF-κB plays on expression of the CYP4F11 gene. We concluded that although there is a significant increase in CYP4F11 expression in the presence of TNF-α, the activation of NF-κB signaling inhibits CYP4F11 expression in a time dependent manner. The expression of CYP4F11 is only significantly increased after 24 hours of treatment with TNF-α; at shorter time points NF-κB signaling overpowers the JNK pathway activation. We believe that these findings may in the future lead to improved drug design for modulating inflammation.

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List of Abbreviations

Cytochrome P450s	CYPs or P450s
Cytochrome P4504F	CYP4F
Leukotriene B4	LTB4
Arachidonic Acid	AA
Interleukin 6	IL-6
Interleukin 1 Beta	IL-1β
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB
Tumor Necrosis Factor-Alpha	TNF-α
Polymorphonuclear Leukocyte	PMNL
5-Lipoxygenase	5-LOX
Cyclooxygenase-2	COX-2
Phosphate-Buffered Saline	PBS
Mitogen activated protein/ERK Kinase Kinase	MEKK
Quantitative Real Time Polymerase Chain Reaction	qRT-PCR
c-Jun N-terminal Kinases	JNK
Microtubule-associated Protein 2	MAP2
Glial Fibrillary Acidic Protein	GFAP
Single Nucleotide Polymorphism	SNP
Prostaglandins	PG

Chapter One: Introduction

Cytochrome P450 Superfamily

Discovery, Nomenclature, Evolution

The Cytochrome P450 (CYPs) family of hemoproteins is the largest and most functionally diverse superfamilies of enzymes. They are responsible for the oxidation, peroxidation, and reductive metabolism of many endogenous compounds such as fatty acids, prostaglandins, leukotrienes, steroids and biogenic amines. A great number of the CYPs are also able to metabolize xenobiotics including drugs, environmental pollutants, natural plant products, and alcohols [1]. The ability for mammalian tissues to oxidize non-polar xenobiotics was known in the early 1950's, however the enzyme or enzymes responsible for the catalysis were not [2]. In 1958 studies from pig and rat microsomal protein fractions noted the existence of a spectral absorbance peak (Soret peak) at 450nm in the presence of carbon monoxide [2]; this was later identified as a P450 hemoprotein [3]. This unique spectral absorbance peak is where CYPs obtain their name.

Human CYPs are named using the following system: CYP, denoting cytochrome P450, an Arabic number designating the P450 family, a letter indicating the subfamily when there are two or more subfamilies known, and then followed by an Arabic numeral representing the individual gene [1]. For example: CYP4F11. If a CYP sequence displays amino acid identity that is greater than 40% they are grouped into the same family [2]. If those sequences are greater than 55% identical then they are placed in the same subfamily, followed by a sequence identity of 97% or higher resulting in alleles [2].

The human genome encodes 57 CYP proteins (http://drnelson.utmem.edu/CytochromeP450.html). A recent survey performed by the Guengerich group classified fifteen CYPs as being involved in the metabolism of xenobiotic chemicals, fourteen being involved in the metabolism of sterols; four that oxidize fat-soluble vitamins; and nine involved in the metabolism of fatty acids and eicosanoids in which for the topic of my dissertation the CYP4F family resides. The remaining fifteen CYPs substrates are undesignated [4].

The first CYP is believed to have been present as early as three and a half billion years ago [2]. The thought is that as the earth's atmosphere accumulated more molecular oxygen CYPs were recruited to protect life forms from oxygen toxicity. There was then a switch wherein metabolism of toxic plant allelochemicals, xenobiotics and, thereby, modern pharmaceuticals arose as an important feature of the cytochrome P450 gene superfamily [2]. It is hypothesized that during evolution there was a "plant-animal warfare," whereby animals evolved new P450s to synthesize the toxic phytoalexins produced by plants [5]. It is this evolution of CYP dependent metabolism of plant metabolites by insects and mammals that is most likely linked to present day role of CYPs ability to detoxify pollutants, toxins and pharmaceuticals [2].

Monoxygenase system

Cytochrome P450s main function is to activate molecular oxygen to yield a reactive species that can attack chemical sites to form hydroxyl groups into structures as unreactive as hydrocarbon chains and aromatic rings [6]. There are three major components to the monoxygenase enzyme system: 1) Heme protein,

CYP, 2) the flavoprotein, NADPH CYP-reductase and 3) phospholipids [7]. The reductase is needed to transfer two electrons to the CYP thus providing reducing equivalents to the heme iron for reduction of molecular oxygen and product formation during catalysis [4]. The basic reaction takes one molecular oxygen atom and inserts it into a substrate (SH), the second atom being reduced to water:

The CYP catalytic cycle follows steps shown in Figure 1.1 [4]. The main steps are

$$\mathbf{NAD(P)H + O_2 + SH + H^+ \longrightarrow NAD(P)^+ + SOH + H_2O}$$

as follows:

1. Substrate binds to CYP recognition site
2. An electron from NADPH via Cytochrome P450 reductase reduces heme ferric iron
3. Oxygen binds the ferrous iron
4. Second electron forms the peroxy iron complex
5. Cleavage of the O-O bond forming H_2O and a reactive iron-oxo complex
6. Oxygen is then transferred to the bound substrate
7. Product is released

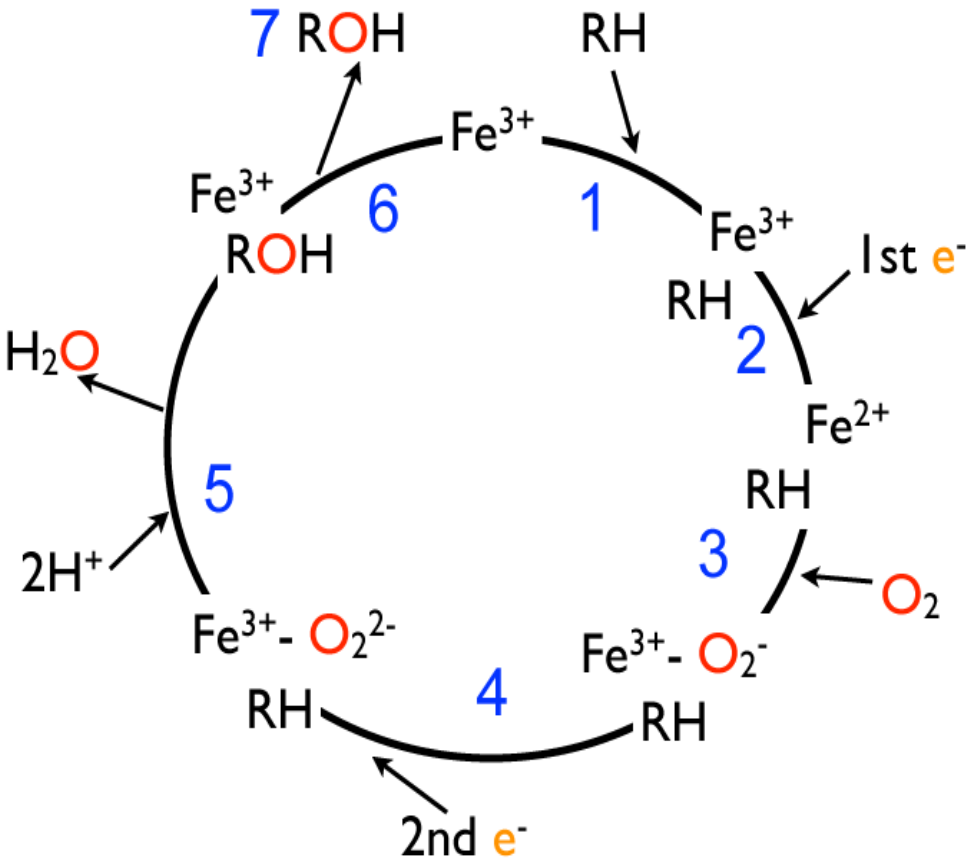


Figure 1.1 Cyctochrome P450 catalytic cycle. The Cytochrome P450 enzymes act as monoxygenase systems, where molecular O_2 is used to oxidize lipophilic substrates (RH). This figure is adapted from the PROMISE mirror database (website: http://metallo.scripps.edu/PROMISE/P450.html)

Hepatic and extra hepatic metabolism

Even though there are many different families of CYPs the conventional wisdom has been that xenobiotics are mainly metabolized by the CYP families CYP1, CYP2, and CYP3 [8]. With current research in our laboratory we have also found that one isoform of the CYP4F family is as good at metabolizing xenobiotics as CYP3A4 the CYP responsible for metabolizing about 30% of clinically available drugs [7]. In mammals, the liver is the major organ with cytochrome P450 expression and activity. However, the presence of phase I metabolizing enzymes, which include CYPs [9],[10],[11] along with phase II metabolizing enzymes [9], [11], and phase III transporters [12],[13], are also expressed across many other tissues. This distribution of metabolizing enzymes also makes them promising candidates for drug delivery. However, most of the studies conducted on phase I CYP enzymes are focused mainly on the CYP families 1,2, and 3, and since our laboratory has uncovered the ability of CYP4F11 to metabolize drugs, we have focused thisP450s research to examine the distribution of the 4F family in the human body and how the CYP4Fs are regulated.

Cytochrome P450s as therapeutic targets

Currently there are several CYPs that have been targets for drug design, chiefly CYP19A1 and CYP5A1. CYP19A1 or aromatase is responsible for a key step in the biosynthesis of estrogens, and it is this function that drug design has been utilized to inhibit its function [14]. Estrogen production is a component of some cancer processes and other diseases and thus the use of this CYP as a drug target has had a great affect on the treatment of estrogen-related diseases.

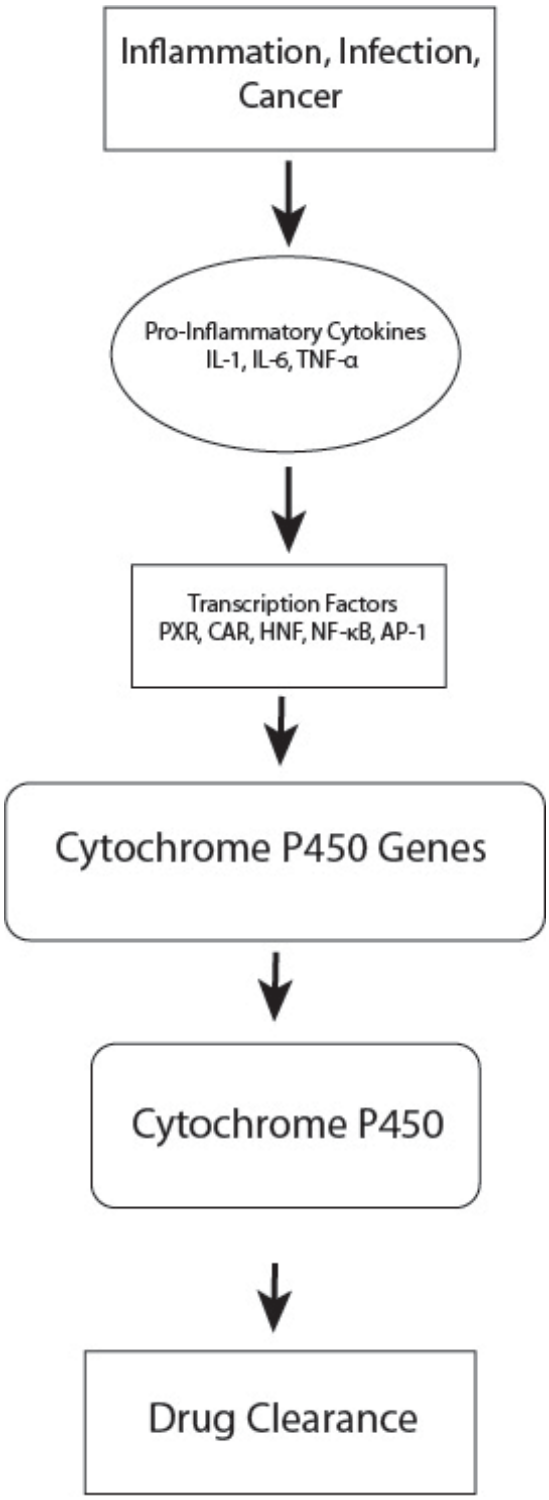
Furthermore, CYP5A1 or thromboxane A synthase specializes in converting prostaglandin H_2 to thromboxane A_2, a potent vasoconstrictor and inducer of platelet aggregation [15]. Drugs designed as thromboxane A synthase inhibitors are valuable as antithrombotic and antihypertensive drugs [16]. For most other CYPs the attempts to inhibit their activities to achieve either greater drug availability, as in the case of CYP3A4 substrates, or to inhibit the progression of a known function, such as synthesis of arachidonic acid, is still in the beginning stages [7]. Nevertheless, since the goal is to serve people with an effective treatment for disease, knowledge of the function, regulation and expression of cytochrome P450s as they regulate drug availability and inflammation is important in obtaining that goal.

Inflammation and Cytochrome P450s

Another complication for drug design is the fact that during many diseases and infections the normal host defense mechanism is activated and mediators of inflammation are present throughout the body. This is a problem because inflammation modifies the expression, activity, and functions of drug-metabolizing enzymes and drug transporters [17]. Mainly, the inflammatory stimuli cause decreased catalytic abilities of CYPs which could lead to the dose-dependent drug toxicity associated with impaired drug clearance [18]. Thus, understanding the role inflammation plays in regulating CYPs is very important for clinical drug therapy in disease states. During an inflammatory response the liver's ability to handle drugs is not optimal and the expression of CYP genes are down-regulated at the transcript levels [18], which leads to the observed decreased catalytic abilities.

Carcillo et al. showed that children with sepsis had a twofold reduction in antipyrine clearance compared to controls [19]. This study shows the importance of inflammatory mediator regulation of a number of CYPs and how marker inflammatory mediators may play a role. In their study they focused on the cytokine Interleukin-6 (IL-6) and nitric oxide (NO) [19]. They concluded that for drugs metabolized by CYPs there needs to be a reevaluation of the use of standard drug dosage schedules especially for patients with sepsis, which show a high increase in host defense inflammation measures [19]. Figure 1.2 is a pathway proposed for the loss of catalytic activity during an inflammatory response [20].

Figure 1.2. Modified proposed pathway for the loss in drug metabolism during an inflammatory response. Diseases cause an inflammatory response that causes the release of cytokines from macrophages, and monocytes which lead to a modified transcription profile of genes that are active in the liver. These changes down-regulate most of the CYP genes expressed in the liver. Which ultimately lead to a low drug clearance and high chance of drug toxicity [20].


Inflammation, Infection, Cancer
Pro-Inflammatory Cytokines
IL-1, IL-6, TNF-α
Transcription Factors
PXR, CAR, HNF, NF-κB, AP-1
Cytochrome P450 Genes
Cytochrome P450
Drug Clearance

An important nuclear transcription factor in the mediation of inflammation is the nuclear transcription factor-kappa B (NF-*κ*B). Two classic cytokines can lead to the release of NF-*κ*B, interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-*α*); stimulation of NF-*κ*B by these two cytokines is considered the classical pathway [21]. This current research project focuses mainly on these cytokines because of their up-regulation during normal host defense processes, and their down stream activation of NF-*κ*B, which also serves as an important transcription factor in the regulation of CYPs. NF-*κ*B regulates over 200 genes involved in a plethora of cellular processes [22],[23],[24],[25]. NF-*κ*B is composed of hetero- or homo-dimers of five members, p52, p50, p65 (RelA), RelB, and c-Rel and each unit has its own biological activity [21]. NF-*κ*B has been reported to mechanistically regulate the transcription of CYPs either by indirectly regulating the gene through mutual repression between NF-*κ*B and many other nuclear receptors, or by directly binding to the response element of the CYP gene [22]. Another mechanism that has also been reported is the ability of NF-*κ*B to affect the stability of the CYP on the protein level [26], [22]. The mechanistic capabilities of NF-*κ*B and its ability to regulate CYPs are important in the scheme of understanding the expression response of CYPs during an inflammatory response so that drug design and patient care can be improved.

Most current studies examining changes observed in CYP expression during normal host defense mechanism response have been performed on CYP Families 1,2 and 3 [20],[27],[19]. Our laboratory is working on the effects that inflammatory mediators and thus host response mediators have on the CYP4F

family and the significance of this information for the future of drug design and resolution of inflammation.

Cytochrome P450 4F subfamily

Function

The Cytochrome P450 4F (CYP4F) family was first discovered by two different groups as part of an effort to define an inactivation pathway for leukotriene B_4 (LTB_4), a powerful mediator of inflammation [28],[29]. The CYP4Fs catalyze ω and ω-1-hydroxylation reactions; it is this reaction that aids in the removal of LTB_4, (Figure 1.3) a prominent proinflammatory signal which, when left unregulated, can cause secondary harmful effects [30].

There are seven CYP4F isoforms presently known to be expressed in humans: CYP4F2, CYP4F3A, CYP4F3B, CYP4F8, CYP4F11, CYP4F12 and CYP4F22. Table 1.1 shows each family members' characteristics. It is important to note that of the seven isoforms the primary drug metabolizer is CYP4F11 **[31]**. However, it is the ability of most of the family members to metabolize multiple mediators of inflammation and regulatory processes such as LTB_4, prostaglandins and arachidonic acid (AA) that has been the focus of the current project on the regulation of these important enzymes.

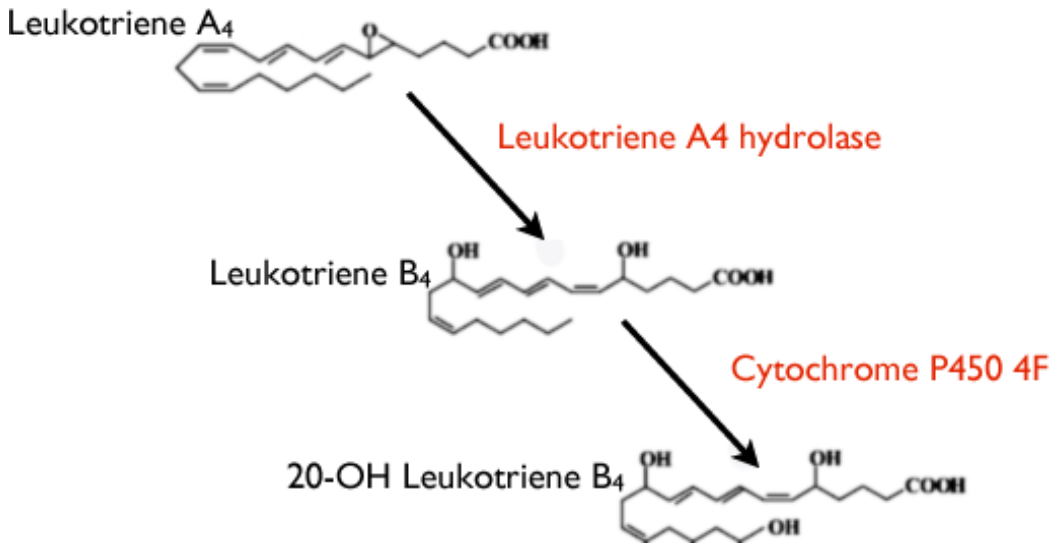


Figure 1.3 Enzymatic pathway for metabolism of LTB_4[32]. After Leukotriene B_4 is produced CYP4Fs are able to start the deactivation process of LTB_4 by hydroxylation.

CYP4F human subfamily members and their substrates

Isoform	Substrates
CYP4F2	LTB4, AA, Vitamin E
CYP4F3	LTB4, AA, HETE
CYP4F8	AA, Prostaglandin H2 analogues
CYP4F11	Erythromycin, Clorpromazine, Theophylline Benzpheamine, Ethylmorphine, AA, LTB4
CYP4F12	Ebastine, AA, Prostaglandin H2 analogues
CYP4F22	-NK-

-NK-: not known

Table 1.1 CYP4F subfamily members and their respective known substrates

Expression and Distribution

There have been a few reports about the location of many different CYP4Fs throughout the human body. CYP4F2 was shown to be expressed in liver, kidney, skin and several other tissues and tumors [32],[33]. CYP4F3 was shown to be expressed in the human liver, while CYP4F8 was shown to be present in seminal vesicles, epidermis, hair follicles, sweat glands, proximal renal tubules, and epithelial linings of the gut and urinary tract [34]. CYP4F11 has been localized to the human liver, kidney, heart, brain and skeletal muscle [35]. Lastly, CYP4F12 has been reported to be expressed in the liver, kidney, seminal vesicles, small intestine, prostate gland and ovarian follicles [36],[37],[38]. While the presence of these isoforms have been identified in a great variety of human tissues, there has not been a complete analysis of the expression level of each isoform of the family as a comparison study to see which isoforms are the predominant members of the family in each tissue. This information would help in understanding the role that the family has in the human body.

Regulation of CYP4Fs during inflammation

Inflammation is known for its contribution to the host defense mechanism where it aids in the fight against infection by a variety of pathogens as well as tissue injury. On the other hand, it also can become diffuse and uncontrolled and thereby produce unwanted complications. The importance of CYP4Fs are shown during this process because after a bacterial infection or tissue injury polymorphonuclear leukocytes (PMNL) or neutrophils are activated and recruited

to the site of inflammation by mediators such as LTB_4 that causes chemotaxis to the site of injury [39],[40]. The CYP4Fs ability to control the inactivation of LTB_4 plays an important role in making sure excess LTB_4 is not in the system, which could lead to unregulated inflammation. Studies examined the changes in CYP4F expression during an inflammatory response to determine if indeed the CYP4Fs have a capability to resolve inflammation [41]. Kalsotra et al. hypothesized that during the pro inflammatory phase of host defense there was a low expression level of CYP4Fs, which begins to show a marked up-regulation of CYP4Fs as the anti-inflammatory phase starts. This elevation of CYP4F activity supports the resolution of the excess of inflammatory mediators [32]. These findings have led our laboratory to study the functions of the specific CYP4F isoforms that are expressed in the human, and examine how they are regulated during an inflammatory response. Our goal is to provide a basis for how these important enzymes work throughout an inflammatory episode. This understanding may in the future lead to better drug design for modulating inflammation.

Chapter Two: Human Distribution, Expression and Inflammatory Mediator Regulation of CYP4Fs

Abstract

Cytochrome P450s (CYP) play a central role in the the metabolism, elimination and detoxification of xenobiotics but many of them play a key role in the metabolism of endogenous compounds. The CYP4F family is instrumental in the inactivation of leukotriene B_4, a potent inflammatory mediator. However, not much is known about the relative expression of each CYP4F isoform in human tissues. In this study we determined the expression profiles of the CYP4F isoforms in the liver, lung, heart, kidney and spleen. We show that during an inflammatory response in tissue samples the profile of the CYP4F isoforms change and that the changes seen are consistent with the presence of inflammatory cytokines. Using the HepG2 human liver cell line we determined that IL-1β, TNFα and IL-6 causes a decrease in CYP4F expression, but that IL-1β and TNFα causes an increase in CYP4F11 expression, which is a different regulatory response from its other family members. Overall, the study confirms what was shown in animal model studies is also seen in human tissues and cells.

Background

Cytochrome P450s (CYP) catalyze lipophilic compounds into compounds that are more soluble. They play a central role in the metabolism, elimination and detoxification of xenobiotics. Studies have recently focused on the extra-hepatic metabolic capacities of these enzymes and shown discrete and different activity profiles for extra hepatic tissues. There is also evidence that the different tissue organs regulate extra-hepatic drug metabolizing enzymes in a specific manner that may be associated with the function of that tissue [9]. Several studies have been performed in animal models to determine the distribution and expression of cytochrome P450s [42][43]. However, it is not always possible to translate animal distribution studies to humans with accuracy and certainty [44]. This lack of close correlation may be due to the differences in the number of cytochromes P450 and isoform specificity from one species to another [45].

Our current study focuses on the CYP4F family of enzymes and their localization and regulation. This CYP4F family was first identified because of the ability to inactivate leukotriene B_4 (LTB_4), a powerful mediator of inflammation [28]. Currently seven identified human isoforms in the CYP4F family have been identified, and in this study we focus on CYP4F2, CYP4F3A, CYP4F3B, CYP4F11 and CYP4F12. These isoforms vary in their substrate specificity. CYP4F2 is known for its ability to metabolize LTB_4 or arachidonic acid (AA) [46]. CYP4F3A and CYP4F3B are splice variants of each other that are able to metabolize LTB_4 and AA [47]. Christmas et al. were able to show that CYP4F3A has a greater ability of metabolizing and deactivating LTB_4 [47] while CYP4F3B is more efficient at

metabolizing AA into 20-hydroxyeicosatetraenoic (20-HETE) [48]. CYP4F11, unlike the other members of the CYP4F family, is efficient at catalyzing xenobiotics with higher affinity than endogenous compounds like LTB_4 and AA [31]. The final isoform in this study is CYP4F12; it is able to metabolize AA and prostaglandins (PG), which may also help in the termination of inflammation [36].

To determine the distribution of CYP4F isoforms in human liver, lung, spleen, heart and kidney, we designed qRT-PCR assays that quantify the mRNA of each of the CYP4F isoform family members. We also examined the role inflammatory mediators play in the expression and regulation of the CYP4F isoforms under i*n vivo* and *in vitro* conditions.

Materials and Methods

Chemicals

IL-1β, TNFα and IL-6 were obtained from Invitrogen Inc. (Carlsbad, CA).

Human Sample RNA

Pooled Human RNA were purchased from Clontech, Mountain View, CA. Human tissue samples from the liver, heart, lung, spleen and kidney were also collected and flash frozen by the Department of Pathology at the University of Texas Health Science Center Houston from Memorial Hermann Hospital. This was done in accordance to IRB approval by the University of Texas Health Science Center Houston. A description of the human samples is presented in Table 2.1.

Cell Culture

HepG2 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown at 37^{o}C in a humidified incubator with 5% CO_2 in Minimum Essential Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), L-glutamine and Pen/strep antibiotics. Cytokine treatments were administered 24 hours after seeding the cells and the cells were treated for 24 hours in each case unless otherwise noted.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Human samples were homogenized in TRIzol reagent (Invitrogen) while cells were rinsed with phosphate-buffered saline (PBS), and then subjected to RNA isolation using TRIzol reagent (Invitrogen). DNase I (Invitrogen) was used to alleviate DNA contamination. Aliquots (100 ng) of total RNA were reverse-transcribed by SuperScript II Reverse Transcriptase (Invitrogen) in triplicate, including a reverse transcription blank to evaluate the presence of contaminating genomic DNA. Amplification was performed using Taq DNA Polymerase (Invitrogen) with an ABI Prism 7700 (Applied Biosystems, Foster City, CA) at 95^{o}C for 1min, followed by 40 cycles at 95^{o}C for 12s and 60^{o}C for 30s. *CYP4F* mRNA levels were measured using standard curves generated by plotting Ct versus the log of the amount of purified amplicon for *CYP4F* (custom synthesis by Invitrogen) (200ag - 2pg). Abundance of human 18S RNA was used as an internal control.

Sample	Tissue Type	Sex	Pool Size	Age	Race	C.O.D.
Clontech	Li	M	1	51	C	Sudden Death
Clontech	Lu	M/F	3	32-61	C	Sudden Death
Clontech	K	F	1	40	C	Sudden Death
Clontech	S	M/F	12	18-54	C	Sudden Death
Clontech	H	M	3	30-39	C	Trauma
H673	Li,Lu,K,S,H	F	1	71	C	CRF, HTN
H678	Li,Lu,K,S,H	M	1	50	C	Li/K Failure
H693	Li,Lu,K,S,H	M	1	58	C	Septic Shock
H696	Li,Lu,K,S,H	F	1	78	C	Septic Shock
H6103	Li,Lu,K,S,H	F	1	63	C	Septic Shock

Li=Liver, Lu=Lung, K=Kidney, S=Spleen, H=Heart, M=Male, F=Female, C=Caucasian, AA=African American,CRF=Cardiorespiratory Failure, HTN=Hypertension

Table 2.1. Total RNA Source Information

Primers and fluorescent probe sequences for CYP4F isoforms and 18S RNA are reported in Table 2.2.

Statistical Analysis

Data are presented as mean ± S.E.M. One-way analysis of variance followed by Dunnett's multiple comparison test was used for the statistical analysis. Statistical differences were considered significant if $P < 0.05$.

Name	Primers/Probes	5'-3' Sequence
CYP4F2	Forward Primer	CCAGAACAGGCCAATCTGAA
	Reverse Primer	CTCCTCAGTCCCACCTCCC
	Probe	ATGCATAGCGGATTGGTGGCTTTCA
CYP4F3A	Forward Primer	TCTGGATGGGCCCCATCT
	Reverse Primer	TTGATGACAGACCGGATGATGT
	Probe	CGTCATCCGTTTTTGCCACCC
CYP4F3B	Forward Primer	GCCCTGGCACGCAATC
	Reverse Primer	GGAGCAAAGAGCACAGGCTT
	Probe	TCCGCATCTTCCACCCCACCTAC
CYP4F11	Forward Primer	CGAAACAGAACTGGTTTTGGG
	Reverse Primer	GGTCAATGTCTTCATGCCCTC
	Probe	AGGGCCTGGTCACTCCCACGG
CYP4F12	Forward Primer	ATGTCGGCCACCTATTCCC
	Reverse Primer	AGGGTGGCATAAAACGATGA
	Probe	TTACGGTATGGCTGGGTCCCATCAT
Human 18S	Forward Primer	GAGGGAGCCTGAGAAACGG
	Reverse Primer	GTCGGGAGTGGGTAATTTGC
	Probe	TACCACATCCAAGGCAGCAGG

Table 2.2. Sequences of oligonucleotides comprising Human CYP4F isoform TaqMan assays

Results

CYP4F Isoform expression is varied across human tissue samples

Our laboratory has previously shown that in the rat the expression of CYP4F isoforms are varied across liver, lung, brain and kidney rat tissues [49]. We designed this current study to determine the expression profile of the various CYP4F isoforms across human liver, lung, heart, kidney, spleen and brain tissue.

Liver

The CYP4F2 isoform was the predominant CYP4Fs being expressed in the human liver with the other isoforms showing minimal expression. The CYP4F isoform expression level ranking is CYP4F2 > CYP4F3B > CYP4F11 > CYP4F12 > CYP4F3A as is represented in Figure 2.1.

Lung

In the human lung the CYP4F isoform distribution was as follows: CYP4F3B> CYP4F12 > CYP4F11 > CYP4F2 > CYP4F3A. CYP4F3B represented a significant large percentage of the CYP4F expression in the human lung, while CYP4F3A had only minimal expression levels. Figure 2.1.

Heart

In the heart the distribution of CYP4Fs follows the order of CYP4F3B > CYP4F12 > CYP4F2 > CYP4F11 ~ CYP4F3A as seen in Figure 2.1

Spleen

The spleen had the following order of expression: CYP4F3B > CYP4F2 > CYP4F12 > CYP4F11 ~ CYP4F3A. The expression in the spleen shows a

representation of mostly CYP4F3B with a smaller expression profile for CYP4F2. There is minimal expression of CYP4F3A, CYP4F11 and CYP4F12.

Kidney

The kidney like the liver was very high in expression of CYP4F2 and CYP4F3B isoforms. The distribution is as follows: CYP4F2 > CYP4F3B > CYP4F11 ~ CYP4F12 > CYP4F3A.

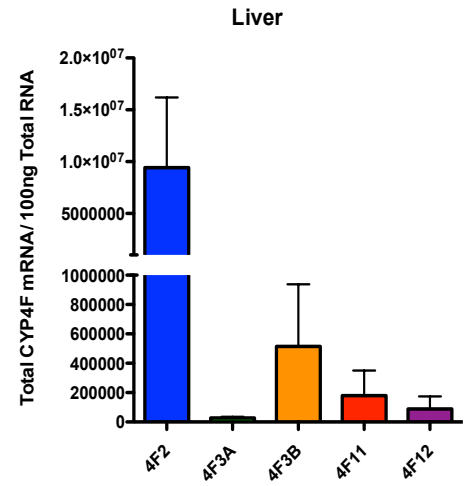
The distribution of CYP4F isoforms in various tissues are represented in the pie chart showing their relative expression (Figure 2.2). This varying degrees of isoform distribution is consistent with the differences in expression levels observed in our previous studies of the rat model [49].

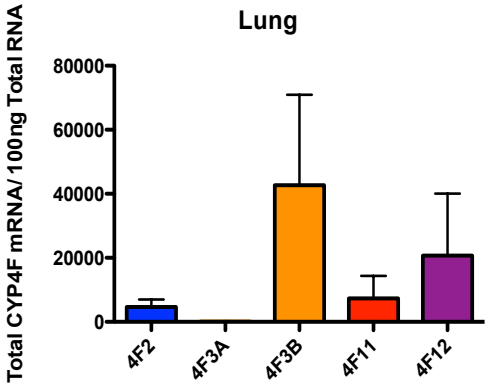
Patient samples with diseases that have an inflammatory component have varied level of CYP4F expression

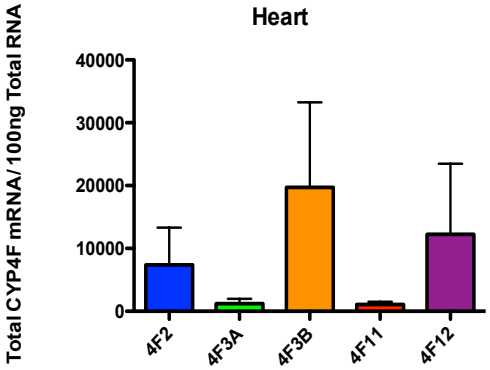
To determine whether inflammation plays a role in the regulation of CYP4Fs we examined patient samples with an inflammatory component implicated in the cause of death. The cause of death was determined by a pathologist using markers of inflammation in histological slides of each tissue. We were able to compare the degree of alteration in CYP4F expression profile occurring in each tissue in comparison to the averaged expression levels for each form in each tissue. Figure 2.3A shows a representative sample of a patient with a disease with an inflammatory component and illustrates the changes in the expression profile of CYP4Fs resulting from inflammation in that tissue. The liver sample from patient H6103 liver had inflammatory markers based on histological markers seen in the slide Figure 2.3B. Increased inflammatory markers are identified as an infiltration of

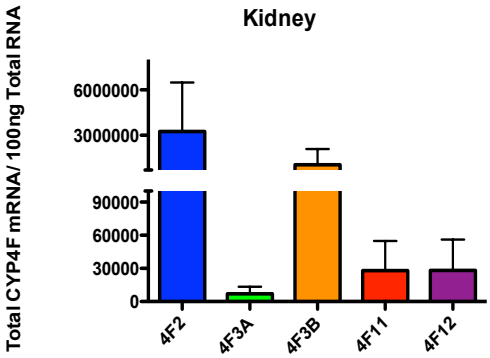
neutrophils and leukocytes. As noted in Table 2.1 the cause of death for patient H6103 was septic shock, which causes the release of a myriad of inflammatory mediators throughout the body. Further, pathological analysis of the liver resulted in an inflammatory rating of two and a diagnosis of hepatitis. When examining the distribution differences between liver sample H6103 and pooled liver we found significant increase in expression of CYP4F11, CYP4F3B, and CYP4F3A isoforms. Figure 2.4A also shows a representative sample of a patient with a disease that included an inflammatory component and the alterations it causes in the expression profile of CYP4Fs. The liver sample from patient H696 liver had increased inflammatory markers such as infiltrated neutrophils and leukocytes, as is seen in Figure 2.4B. As noted in Table 2.1 patient H696 died of septic shock as well, and the spleen was given an inflammatory rating of two with a reactive hyperplasia and the presence of hemorrhage and white pulps. When examining the distribution differences between sample H696 and pooled liver we observed significant increase in CYP4F11, CYP4F2, and CYP4F3A isoform expression.

Figure 2.1. Transcript Copy Numbers of CYP4F Isoforms Across Different Tissues. Total RNA from samples listed in Table 2.1 were used to asses the mRNA transcript numbers of CYP4F isoforms in the liver, lung, heart, kidney and spleen. CYP4F2 and CYP4F3B shows the highest level of transcript copies across most tissues. CYP4F3A, CYP4F11 have minimal expression across varied tissues and CYP4F12 showed differences from high in the lung to low out of most other tissues.


Liver
Total CYP4F mRNA/ 100ng Total RNA
2.0×10^07
1.5×10^07
1.0×10^07
5000000
1000000
800000
600000
400000
200000
0
4F2
4F3A
4F3B
4F11
4F12


Lung
Total CYP4F mRNA/ 100ng Total RNA
80000
60000
40000
20000
0
4F2
4F3A
4F3B
4F11
4F12


Heart
Total CYP4F mRNA/ 100ng Total RNA
40000
30000
20000
10000
0
4F2
4F3A
4F3B
4F11
4F12


Kidney
Total CYP4F mRNA/ 100ng Total RNA
6000000
3000000
90000
60000
30000
0
4F2
4F3A
4F3B
4F11
4F12

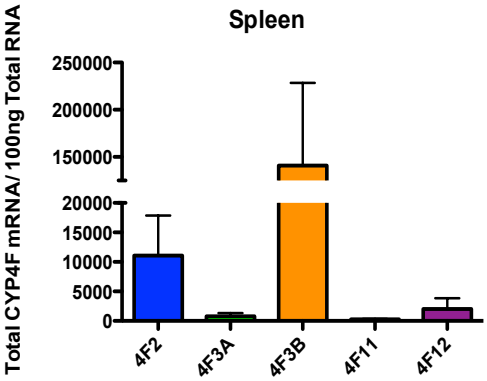

Spleen
Total CYP4F mRNA/ 100ng Total RNA
250000
200000
150000
20000
15000
10000
5000
0
4F2
4F3A
4F3B
4F11
4F12

Figure 2.2 Isoform and Tissue Specific CYP4F Distributions.

Total RNA from samples listed in Table 2.1 were prepared and assayed using qRT-PCR as described in the Methods and Materials section. The pie charts represent the relative distribution of CYP4F isoforms in the liver, lung, kidney, heart and spleen.

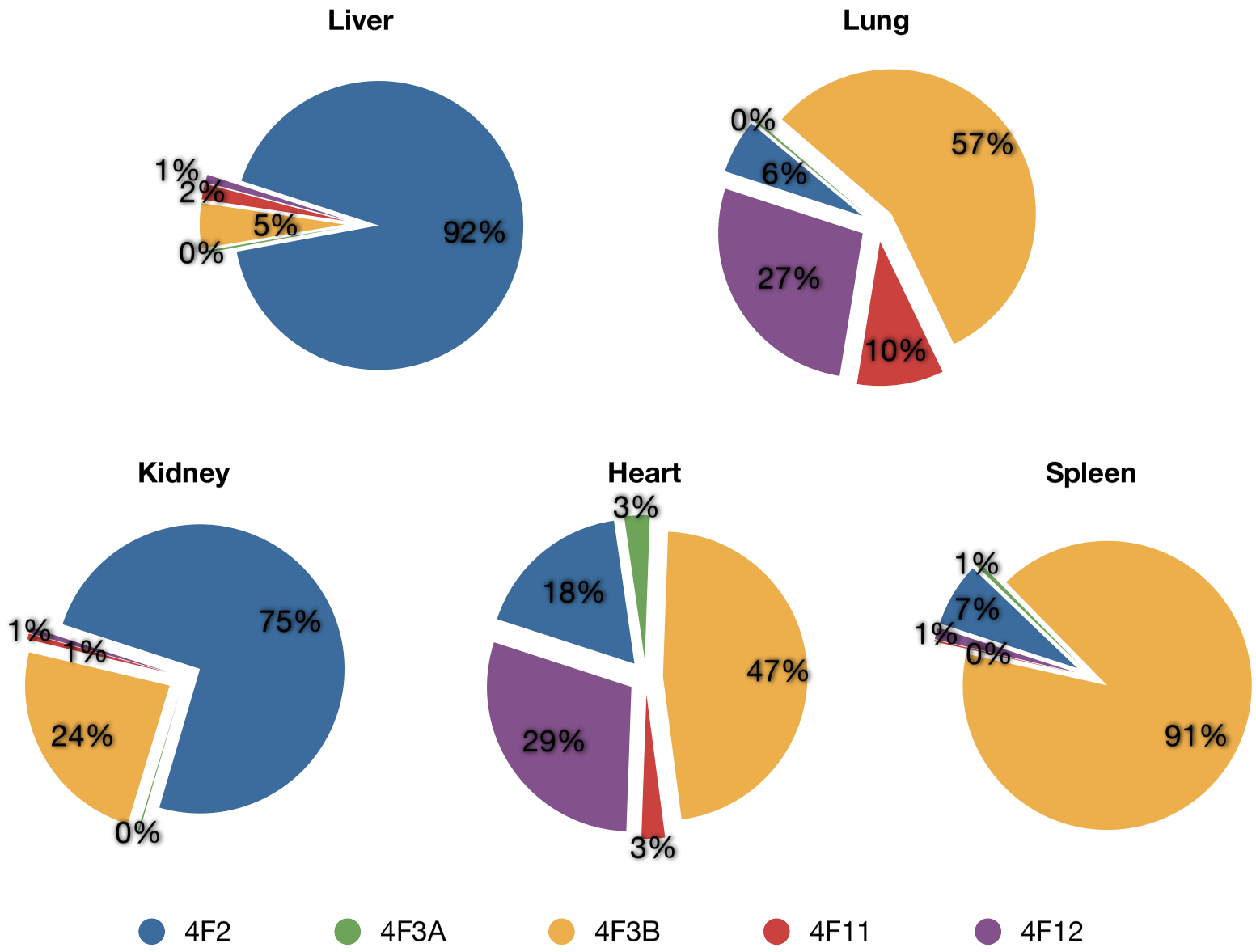
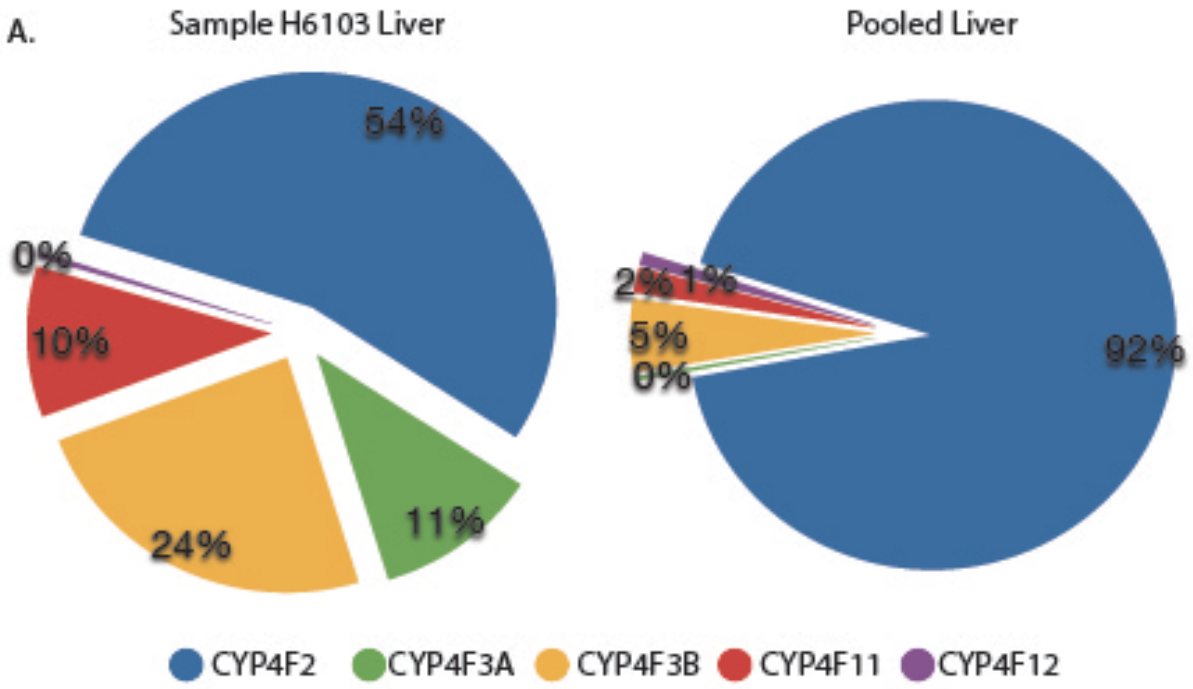

Liver
92%
1%
2%
5%
0%
Lung
0%
57%
6%
27%
10%
Kidney
75%
1%
1%
24%
0%
Heart
3%
18%
47%
29%
3%
Spleen
1%
7%
1%
0%
91%
4F2
4F3A
4F3B
4F11
4F12

Figure 2.3. Sample 103 Inflamed Liver Compared to Pooled Liver.

A) Sample 103 inflamed liver that was diagnosed with hepatitis was used to compare any changes seen in CYP4F isoforms in a diagnosed inflammatory tissue. There was an increase in CYP4F11, CYP4F3A, and CYP4F3B while there was a reduction in CYP4F2 expression. B) A representative histological slide of sample 103's liver with an increase of infiltrated neutrophils which represents the presence of inflammation.


A.
Sample H6103 Liver
Pooled Liver
54%
0%
10%
24%
11%
2%
1%
5%
0%
92%
CYP4F2
CYP4F3A
CYP4F3B
CYP4F11
CYP4F12

B.

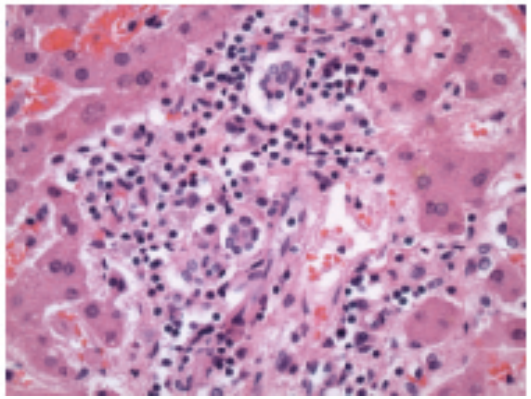
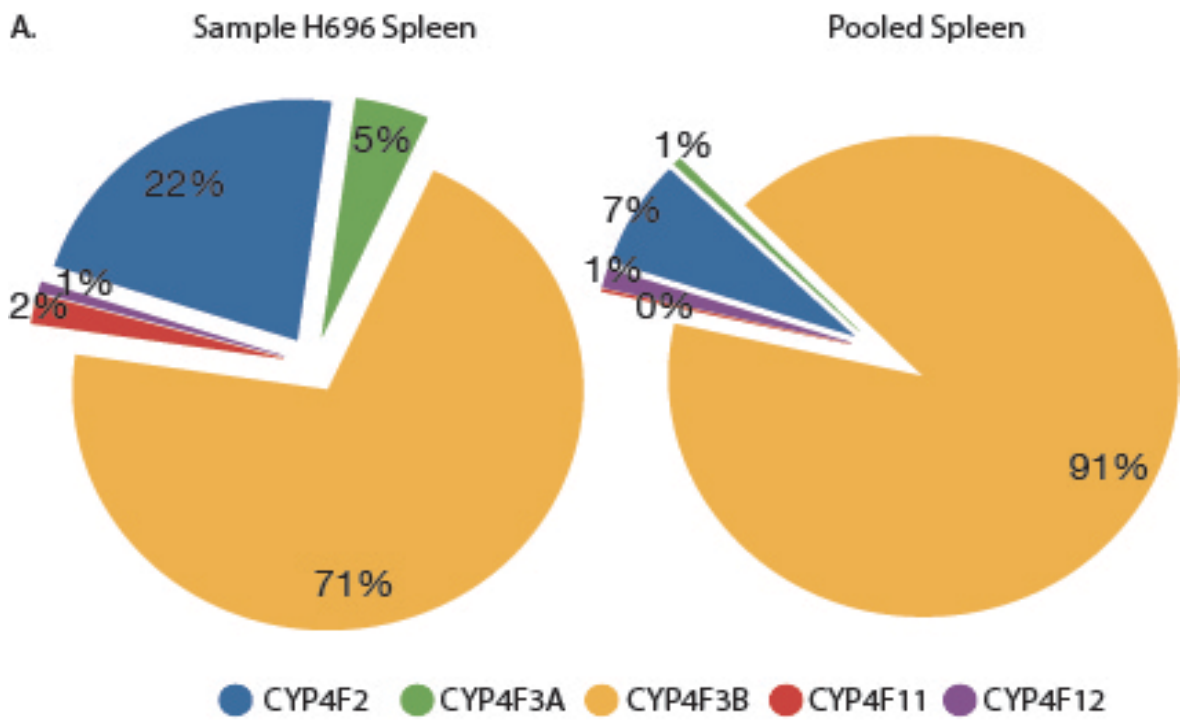
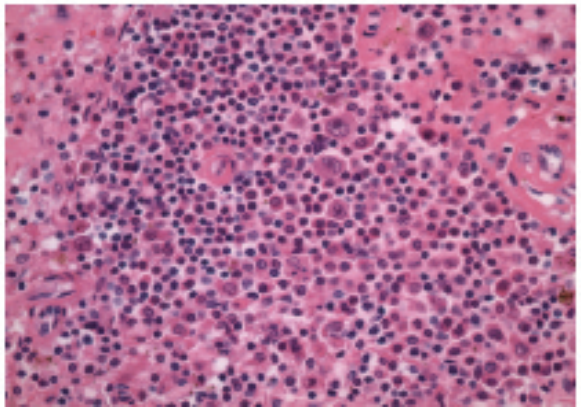


Figure 2.4. Sample 96 Inflamed Spleen Compared to Pooled Spleen.

A) Sample 96s' inflamed spleen that was diagnosed with reactive hyperplasia was used to compare any changes seen in CYP4F isoforms in a diagnosed inflammatory tissue. There was an increase in CYP4F11, CYP4F3A, and CYP4F2 while there was a reduction in CYP4F3B expression. B) A representative histological slide of sample 96s' spleen with an increase of infiltrated neutrophils which represents the presence of inflammation.


A.
Sample H696 Spleen
Pooled Spleen
22%
5%
1%
2%
71%
1%
7%
1%
0%
91%
CYP4F2
CYP4F3A
CYP4F3B
CYP4F11
CYP4F12

B.

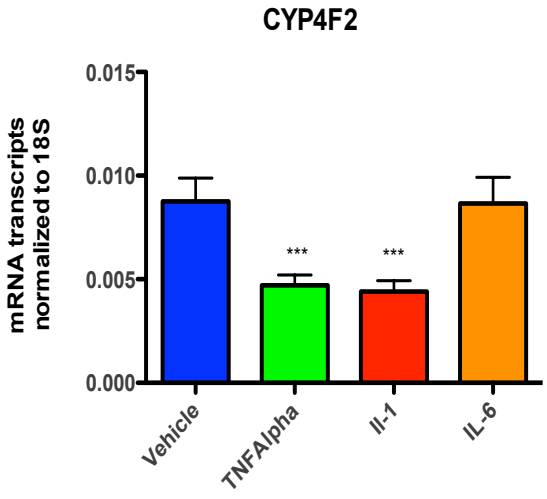


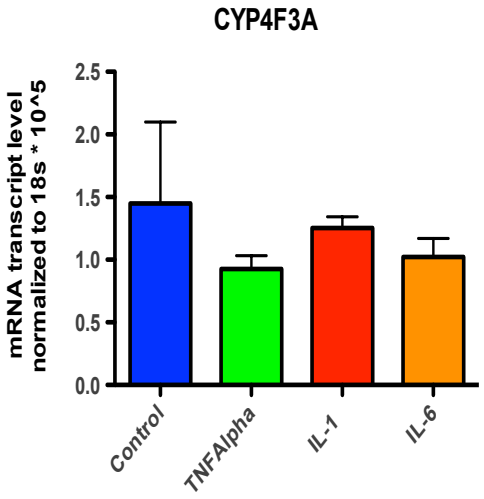
Specific cytokines can change CYP4F isoform expression in HepG2 cells

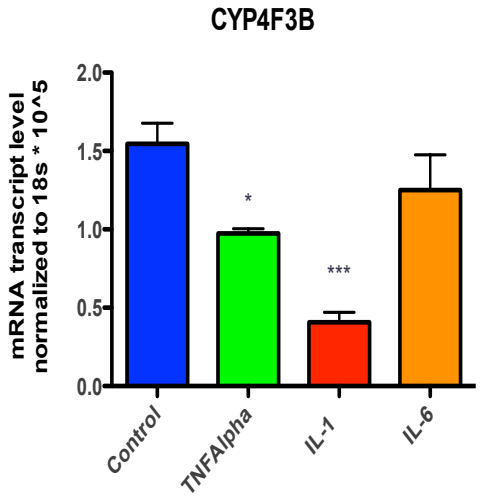
To determine whether if proinflammatory cytokines can regulate the changes seen in CYP4F isoforms we conducted a 24-hour treatment study on HepG2 cells with cytokines and measured the levels mRNA transcripts of each CYP4F isoform. We tested IL-1β, TNFα and IL-6 cytokines and found that in most cases IL-1β and TNFα caused a down regulation of CYP4F isoforms (CYP4F2, CYP4F3B, and CYP4F12) while IL-6 seemed to play little to no role in regulating the CYP4Fs. CYP4F11 however showed an increase in transcript levels in the presence of both IL-1β and TNFα. The ability to change CYP4F expression profiles in a controlled environment provides more evidence that indeed the inflammatory response in human tissues induce changes to these enzymes that metabolize xenobiotics endogenous compounds.

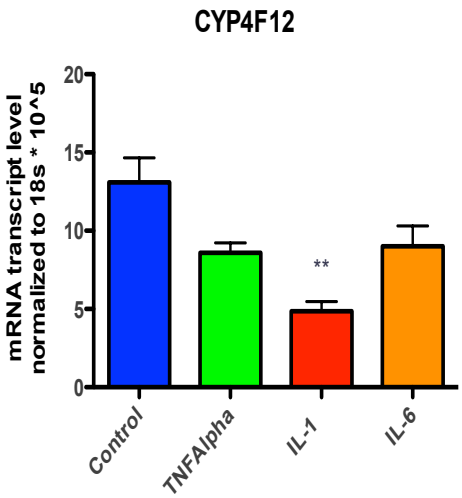
Figure 2.5. Cytokine Regulation of CYP4F Isoforms in HepG2 cells.

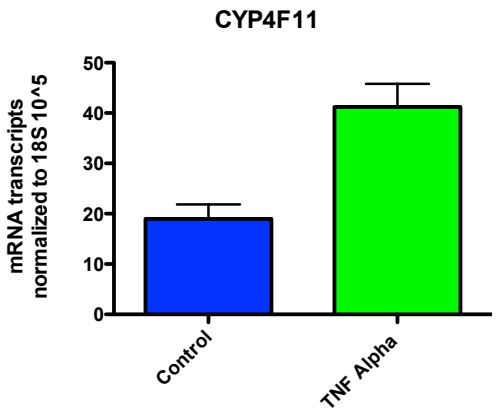
HepG2 cells were plated then treated for 24 hours with either Vehicle, IL-1β, TNFα or IL-6 (10ng/ml). Total RNA was collected after the 24 hour time-period and qRT-PCR was used to quantify the specific isoform expression amount. IL-1β and TNFα were able to change the expression profiles of CYP4F2, CYP4F3B, CYP4F12 and CYP4F11. IL-6 did not vary much from control levels.

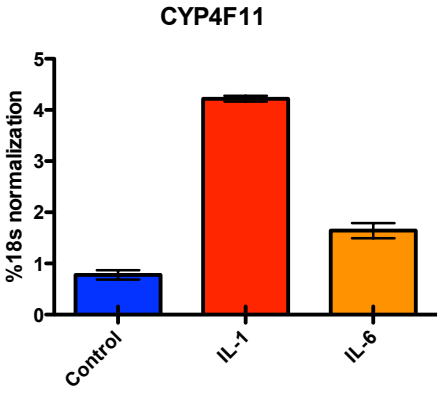

CYP4F2
mRNA transcripts normalized to 18S
0.015
0.010
0.005
0.000
***
***
Vehicle
TNFAlpha
Il-1
IL-6


CYP4F3A
mRNA transcript level normalized to 18s * 10^5
2.5
2.0
1.5
1.0
0.5
0.0
Control
TNFAlpha
IL-1
IL-6


CYP4F3B
mRNA transcript level normalized to 18s * 10^5
2.0
1.5
1.0
0.5
0.0
*
***
Control
TNFAlpha
IL-1
IL-6


CYP4F12
mRNA transcript level normalized to 18s * 10^5
20
15
10
5
0
**
Control
TNFAlpha
IL-1
IL-6


CYP4F11
mRNA transcripts normalized to 18S 10^5
50
40
30
20
10
0
Control
TNF Alpha


CYP4F11
%18s normalization
5
4
3
2
1
0
Control
IL-1
IL-6

Discussion

Phase I metabolizing enzyme research has focused on the liver as the preferred site of experiments because of its known role as the principle organ of biotransformation [50]. It is also believed that xenobiotics are mainly metabolized by cytochrome P450 (CYP)1,2 and 3 family members, and that the other family members focus mainly on endogenous compound trasformation [8]. There has been extensive work done on the regulation, expression profile and function of these family members [43][51-53]. Though much research has focused on the liver because of its functional role in biotransformation, every major tissue has some ability to metabolize xenobiotics [50]. Knowing this information is important because when considering drug disposition the total effect must determined; thus the additive effect of all tissues is important [54]. Many studies have examined the expression and localization of members of the CYP family of genes. Five members of the CYP2C family have been examined by Tsao et al. and they found that there were varied expression profiles of the genes across the murine model [55]. They determined that the sites of these enzymes' expression also play a functional role in that specific tissue [55]. Ding et al. published a review that examined the CYP1, 2 and 3 families to identify their presence in different parts of the respiratory and gastrointestinal tracts [56]. Another study determining the expression and localization of the CYP3A subfamily found that the genes were expressed at different levels across different tissues and suggested that this was due to tissue specific regulation of the promoter region [57]. There is a limited amount of information about the distribution and localization of the CYP4F family in humans.

We undertook to examine the liver, lung, heart, kidney and spleen of a variety of human samples and determine the averaged mRNA expression of the CYP4F isoforms using the highly sensitive technique qRT-PCR. We found that CYP4F2 and CYP4F3B were the most expressed CYP4F isoforms across the different human tissues. CYP4F2, which is responsible for the inactivation of LTB_4 and metabolizes AA into 20-HETE, was highly expressed in the liver and the kidney. Jin et al., reported that the Chinese Human Liver Proteome Profiling Consortium had obtained 42% CYP4F2 and 29% CYP4F3 relative protein abundance in the liver as compared with CYP3A4 [58]. This finding is consistent with the mRNA levels that we have seen in the liver, and they are in the correct order of relative abundance. It is also important to note that the high levels of CYP4F2 and CYP4F3B in the kidney can produce 20-HETE that helps modulate vascular tone and renal tubular function [59, 60]. CYP4F3A was the original CYP4F3 found in neutrophils and is known for its ability to inactivate LTB_4, and CYP4F3B was found to be highly present in liver and kidney using northern blot techniques [48]. We also found that there was a high level of CYP4F12 transcripts in the heart and lung, and very little in the other tissues. It has been reported that CYP4F12 protein is expressed in the liver and kidney in small amounts, but that the highest expression of CYP4F12 protein was in the seminal vesicles and prostate gland [38]. They reported little to no CYP4F12 protein in the aorta and no information was reported about the lung [38]. Thus, further studies would have to be performed to see precise localization of the CYP4F12 protein to determine whether the mRNA expression levels

correlate with protein levels. Overall, CYP4F11 showed small amounts of expression across all the tissues tested.

This study determined the constitutive levels of Human CYP4F isoforms so that there could be a comparison of what these levels are during an inflammatory response. Many studies have reported that inflammatory mediators can cause a change in the expression profile of CYPs [20, 27, 61-63]. The idea that the same down-regulation that occurs in other CYPs could effect the CYP4F family is mainly based on animal model studies done in our lab and other laboratories that show that after injury and the pro-inflammatory stage there is a down-regulation of CYP4Fs. This down-regulation is then reversed in the pro-inflammatory stage of healing where there is an increase in CYP4Fs to help in resolving the inflammatory mediators such as LTB_4 [64-67]. To study this we compared known inflamed tissues defined by histological analysis from patient samples and compared them to our pooled tissue samples. We found that there is a change in CYP4F expression profile in the presence of inflammatory mediators. Of importance is the higher expression of CYP4F3A seen in these samples, which we believe is correlative to the presence of neutrophils at the site of injury/inflammation. However, the changes seen in the CYP4Fs maybe based on a number of signaling processes that need to be studied in a different system, which is why we went to cell culture to identify signaling pathways for changes we saw in the CYP4F expression.

During an inflammatory response or injury a number of cytokines are released as part of the host response [68]. We chose to determine how IL-1β,

TNFα and IL-6 would effect the expression of the CYP4Fs. We found that for a majority of the CYP4Fs, IL-1β and TNFα treatment caused down-regulation of mRNA CYP4F transcripts except for that of CYP4F11. IL-6, however, seems to have little to no effect on the expression of the CYP4Fs. We also found that there was no significant difference between cytokine-treated and control CYP4F3A expression. The conclusion that CYP4F11 can be up-regulated during an inflammatory response and its ability to metabolize xenobiotics could potentially be beneficial to pharmaceutical companies looking for a way to control unwanted inflammatory mediators in tissues. Understanding more about the regulatory controls of the CYP4F family and their ability to resolve inflammation, although still in its infancy stage, could become a major help in treating patients.

Chapter Three: Isoform, Ethnicity and Sex Specific Expression Differences in Human Orbital Frontal Cortex

Abstract

Cytochrome P450s play an important role in the brain. They are responsible for metabolism of neurosteroids, neurotransmitters,and xenobiotics in the brain. This study determined the expression of the isoforms of CYP4F family of enzyme in the orbital frontal cortex. We found that CYP4F11, an enzyme that metabolizes xenobiotic and endogenous compounds was expressed at a significantly higher quantity than its other family members. Immuno-histochemical studies revealed that the CYP4F11 protein is localized in neuron and glial cells and that expression of CYP4F11 is not ubiquitous across all cells. We also found that while gender does not affect the CYP4F isoform expression in the orbital frontal cortex, ethnicity does. African Americans express CYP4F11 at a significantly higher rate than their Caucasian counterparts. Overall, this study is the first demonstration of the importance of CYP4F11 expression in brain.

Background

Extensive work has been done on the metabolism of xenobiotics in the liver and extra-hepatic tissues. This is because xenobiotics are found in all facets of our environment, they range from the drugs we take to the air we breath to the food we eat. Cytochrome P450s (CYP) have been identified as the key enzyme that starts the detoxification process for these compounds by transforming them from lipophilic to more polar hydrophilic metabolites. Because xenobiotics come in a variety of shapes and sizes, the CYP enzymes have been located in all tissues and consist of a diverse number of enzymes within its superfamily. Although there has been extensive research on the CYP expression in the liver and other extra-hepatic tissues the brain has special properties that make it unique. One of the main characteristics that is unique to the brain is the presence of the blood brain barrier (BBB). The BBB is made up of 3 cellular components: endothelial cells, astrocyte feet and pericytes [69]. The BBB is further characterized by the presence of tight junctions which help create a selective barrier to prevent the transport of hydrophilic molecules across the BBB; however, small lipophilic compounds and molecules can cross the barrier [69]. The astrocyte end feet that cover 90% of the endothelial cell surface can also release chemical factors and signals that can regulate the permeability of the endothelium [70]. The brain's limited ability to regenerate and the lipophilic characteristics of xenobiotics leaves the brain vulnerable to damage by toxic compounds. Thus the presence of CYPs to metabolize any lipophilic compound that enters the brain is of great importance in detoxification and therefore protection of the brain.

Many of the CYP family members have been identified in the brain, and they include CYP subfamilies 1, 2, 3 and 4 [71]. What many researchers have found is that the expression levels of CYPs in the brain are about 1-10% of that in the liver [72, 73]. Although there is less representation of CYPs in the brain, the expression of most are in specific regions of the brain, and some members of the CYP family of enzymes also show specific splice variants in the brain.

Although CYPs play a role in catalyzing the detoxification of xenobiotics crossing the BBB, a greater majority of CYPs in the brain specialize in the formation of biologically important lipid signaling molecules such as eicosanoids and steroid hormones. These molecules include: estrogen, corticosteroids, bile acids, vitamin D, and androgens [74]. Another important function of CYPs in the brain is the synthesis of 20-hydroxyeicosatetraenoic acid (20-HETE) from aracidonic acid (AA). This activity is important because 20-HETE plays a role in regulating cerebral blood flow and helps in the vasodilation actions of nitric oxide in cerebral circulation [75]. Studying CYP4F expression in the brain is of great importance because of this last point. Many of the isoforms in the CYP4F family metabolize AA into 20-HETE and to know their expression patterns in the brain helps us understand more about how the brain is functioning. This current study was planned to identify which CYP4F isoforms are expressed in the brain and identify cell types, whether it be neuronal or glial, that express the CYP4Fs.

Materials and Methods

Human Sample RNA

Brain samples were collected from the National Institutes of Health by Dr. Thomas Hyde in accordance to NIH IRB approval of human samples. All samples were from the orbital frontal cortex, and sample descriptions are listed in Table 3.1.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Human samples were homogenized in TRIzol reagent (Invitrogen) while cells were rinsed with phosphate-buffered saline (PBS), then subject to RNA isolation using TRIzol reagent (Invitrogen). DNase I (Invitrogen) was used to alleviate DNA contamination. Aliquots (100 ng) of total RNA were reverse-transcribed with SuperScript II Reverse Transcriptase (Invitrogen) in triplicate, including a reverse transcription blank to evaluate presence of contaminating genomic DNA. Amplification was performed using Taq DNA Polymerase (Invitrogen) with an ABI Prism 7700 (Applied Biosystems, Foster City, CA) at 95^{o}C for 1min, followed by 40 cycles at 95^{o}C for 12s and 60^{o}C for 30s. *CYP4F* mRNA levels were measured using standard curves generated by plotting Ct versus the log of the amount of purified amplicon for *CYP4F* (custom synthesis by Invitrogen) (200ag - 2pg). Abundance of human 18S RNA was used as an internal control. Primers and fluorescent probe sequences for CYP4F isoforms and 18S RNA are reported in Table 3.2.

Sample	BrainRegion	AGE (yr)	Gender	Ethnicity	PMI (hr)
910	Orbital Frontal Cortex	52	F	AfAm	10
922	Orbital Frontal Cortex	51	M	Cauc	30.5
942	Orbital Frontal Cortex	61	M	Cauc	19.5
1007	Orbital Frontal Cortex	57	M	AfAm	25
1051	Orbital Frontal Cortex	57	F	AfAm	19
1053	Orbital Frontal Cortex	40	F	AfAm	25
1054	Orbital Frontal Cortex	58	M	AfAm	28.5
1056	Orbital Frontal Cortex	48	M	AfAm	37
1099	Orbital Frontal Cortex	67	F	AfAm	34
1109	Orbital Frontal Cortex	59	F	Cauc	23
1127	Orbital Frontal Cortex	43	F	AfAm	72
1275	Orbital Frontal Cortex	60	M	AfAm	13
1276	Orbital Frontal Cortex	24	M	AfAm	43.5
1314	Orbital Frontal Cortex	53	F	AfAm	50.5
1443	Orbital Frontal Cortex	56	M	Cauc	29.5
1458	Orbital Frontal Cortex	34	M	Cauc	13.5
1473	Orbital Frontal Cortex	44	M	AfAm	29
1522	Orbital Frontal Cortex	64	F	AfAm	52.5
1525	Orbital Frontal Cortex	46	M	AfAm	13

F=Female, M=Male, AfAm=African American, Cauc=Caucasian, PMI=Post-Mortem Interval

Table 3.1. Total Brain RNA Characteristic Information

Name	Primers/Probes	5'-3' Sequence
CYP4F2	Forward Primer	CCAGAACAGGCCAATCTGAA
	Reverse Primer	CTCCTCAGTCCCACCTCCC
	Probe	ATGCATAGCGGATTGGTGGCTTTCA
CYP4F3A	Forward Primer	TCTGGATGGGCCCCATCT
	Reverse Primer	TTGATGACAGACCGGATGATGT
	Probe	CGTCATCCGTTTTTGCCACCC
CYP4F3B	Forward Primer	GCCCTGGCACGCAATC
	Reverse Primer	GGAGCAAAGAGCACAGGCTT
	Probe	TCCGCATCTTCCACCCCACCTAC
CYP4F11	Forward Primer	CGAAACAGAACTGGTTTTGGG
	Reverse Primer	GGTCAATGTCTTCATGCCCTC
	Probe	AGGGCCTGGTCACTCCCACGG
CYP4F12	Forward Primer	ATGTCGGCCACCTATTCCC
	Reverse Primer	AGGGTGGCATAAAACGATGA
	Probe	TTACGGTATGGCTGGGTCCCATCAT
Human 18S	Forward Primer	GAGGGAGCCTGAGAAACGG
	Reverse Primer	GTCGGGAGTGGGTAATTTGC
	Probe	TACCACATCCAAGGCAGCAGG

Table 3.2. Sequences of oligonucleotides comprising Human CYP4F isoform TaqMan assays

Immunohistochemical Analysis

Tissue fixation and immunohistochemistry solvents were all certified histological grade and obtained from Fisher Scientific (Pittsburgh, PA). Brain samples were fixed in 10% formalin solution, after surgical extraction by our neuropathologist colleague from Memorial Herman Hospital. Samples were then fixed being dehydrated in a series of ethanol (50%, 70%, 95%, 100%) and xylene solutions (100%), then embedded in paraffin wax. The wax block molds of brain tissue were then sectioned and mounted onto poly-L-lysine coated glass slides. For immunohistochemical analysis, the slides were rehydrated and then taken through an antigen retrieval process. The tissue samples were incubated at 4^{o}C overnight in primary antibody solution, then incubated for at least 1 hour in secondary antibody solution. The primary antibody solution contained antibody to CYP4F11 (1:100) and antibody to either MAP2 (1:500), or GFAP (1:1000). The secondary antibody solution was either AlexaFluor-594 anti-rabbit (1:250) or AlexaFluor-488 anti-mouse (1:250). The slides were also treated with ProLong Gold Antifade Reagent that contained DAPI to stain for cell nuclei. The slides were then visualized on a fluorescence microscope and images were captured using SPOT RT Slider imaging system. The background color of the microscope images was adjusted using Adobe Photoshop.

Statistical Analysis

Data are presented as mean ± S.E.M. One-way analysis of variance followed by Dunnett's multiple comparison test was used for the statistical analysis. Statistical differences were considered significant if $P < 0.05$.

Results

Cytochrome P450 4F11 is expressed highly in the Orbital Frontal Cortex.

To understand the expression levels of the CYP4Fs in the orbital frontal cortex of the human brain we performed qRT-PCR using specific primer and probe sets that distinguish between each isoform in the CYP4F family. 100ng of RNA from each sample was analyzed and plotted in Figure 3.1. We found that CYP4F11 is the most highly expressed CYP4F isoform in the orbital frontal cortex. There was little to no expression of CYP4F2 and CYP4F3B in the brain samples. CYP4F3A was the next most abundant isoform expressed followed by CYP4F12. Numerically, CYP4F11 represented 88% of the CYP4F family presence in the orbital frontal cortex and had 20000-25000 transcripts per 100ng of RNA. The order of relative abundance is described as CYP4F11 > CYP4F3A > CYP4F12 > CYP4F3B ~ CYP4F2

Gender does not affect CYP4F expression in brain

Studies have shown that gender can be a factor in the expression of CYP enzymes [76, 77]. To elucidate whether gender plays a role in CYP4F expression in brain, the data obtained from male (n=11) and female (n=8) samples was compared and demonstrated that gender does not play a significant role in the expression of CYP4Fs in the orbital frontal cortex (Figure 3.2).

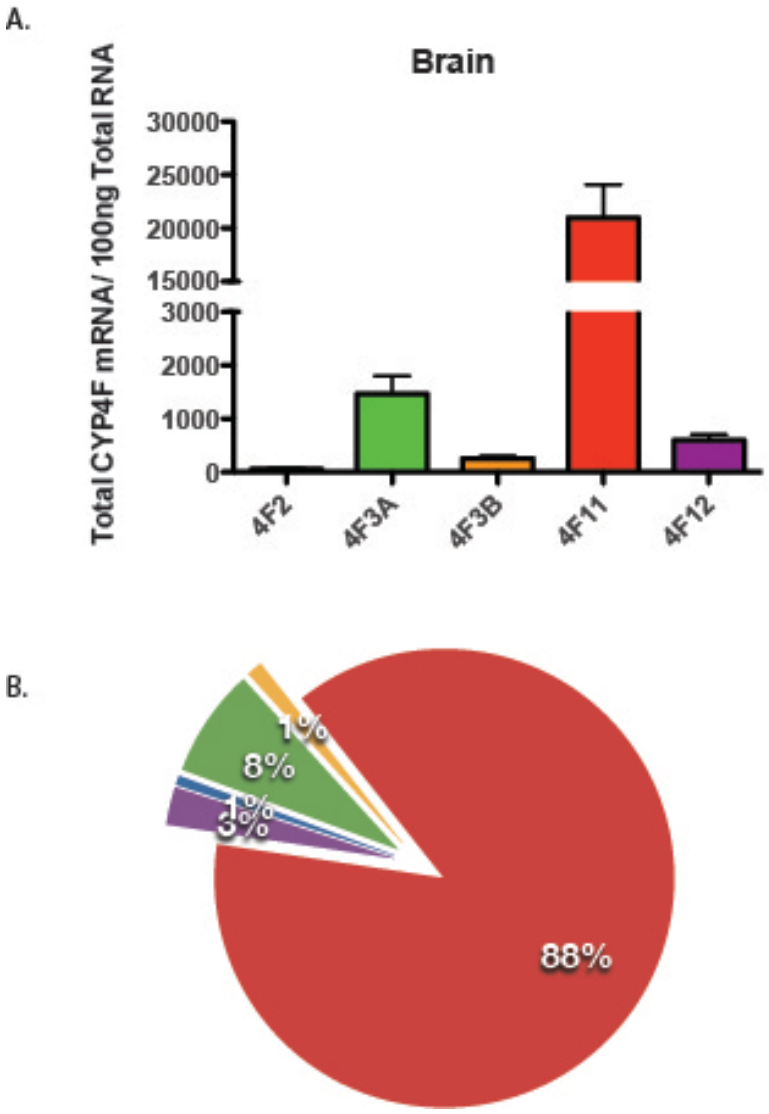


Figure 3.1. CYP4F Isoform Transcript Copy Numbers In Human Brain. A) Total RNA from samples listed in Table 3.1 were used to asses the mRNA transcript numbers of CYP4F isoforms in the brain. B) CYP4F11 shows the highest level of transcript copies compared to other CYP4F's.

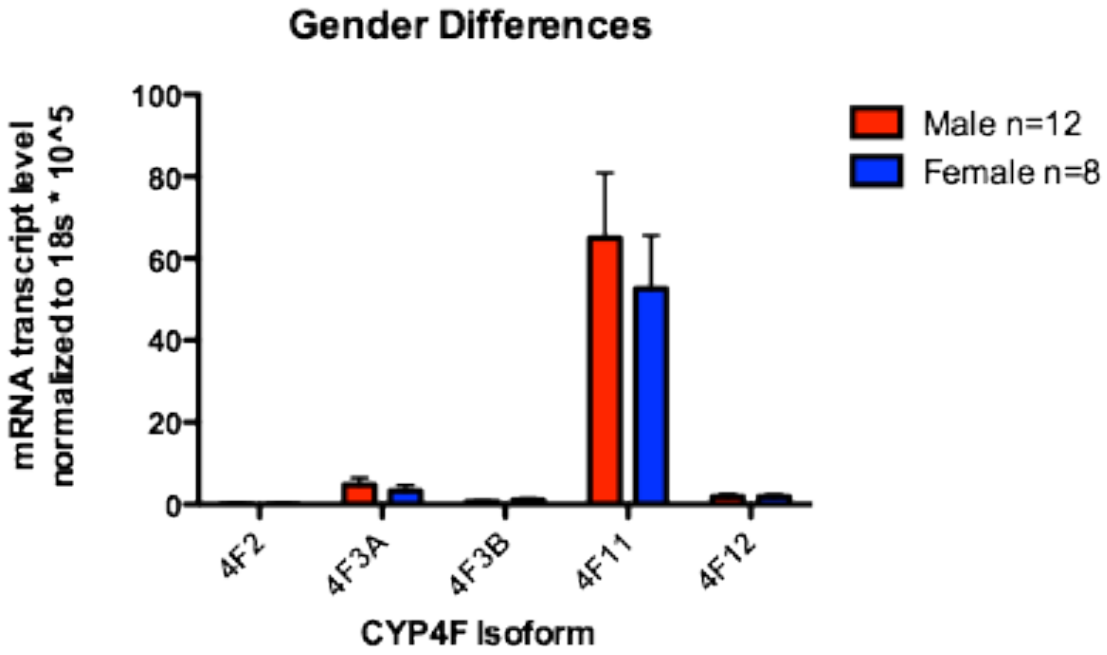


Figure 3.2 Gender differences of CYP4F expression are not observed In orbital frontal cortex.

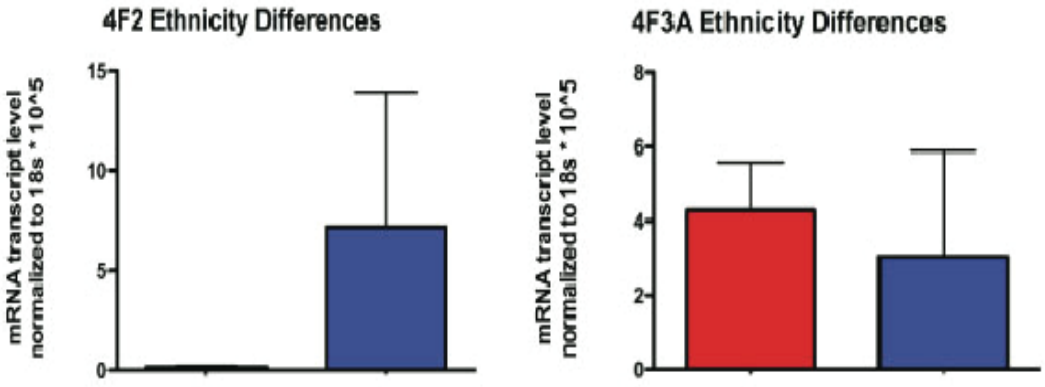
qRT-PCR was used to obtain transcript copy numbers for each CYP4F isoform and compared between male and female samples to determine if gender has an affect on CYP4F expression in the brain. We determined that there were no significant gender differences seen in any CYP4F isoform's expression in the brain.

CYP4F11 expression is more abundant in African Americans compared to Caucasians

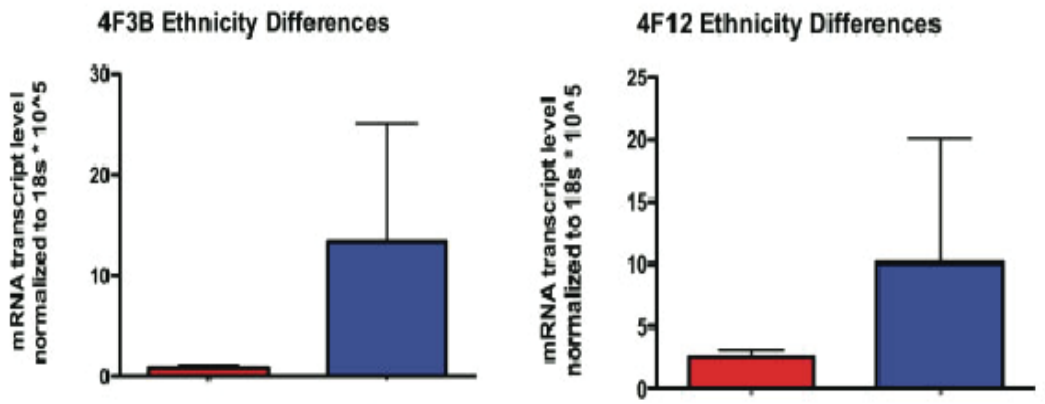
There is growing evidence that ethnicity may play a role in the expression of CYP enzymes [78-81]. To determine if ethnicity was a factor in the expression of the CYP4F family of enzymes we analyzed the expression of each isoform in the orbital frontal cortex of each of our samples. We then compared the African American samples (n=14) to the Caucasian samples (n=5) for each isoform and determined differences if any in the expression of the enzyme (Figure 3.3). We found that CYP4F11 is expressed in high quantities in the African American population as compared to the Caucasian population. CYP4F2 on the other hand showed a greater presence in the Caucasian population as compared to the African American population where the expression of CYP4F2 was either low or absent in the sample population. However, due to the variance in the Caucasian population this finding was not significant at the $P<0.05$ level. This trend was also seen in the expression of CYP4F3B and CYP4F12. CYP4F3A, however, showed few differences in expression between the two ethnic populations.

Figure 3.3 Ethnicity Differences of CYP4F Isoforms In Human Brain.

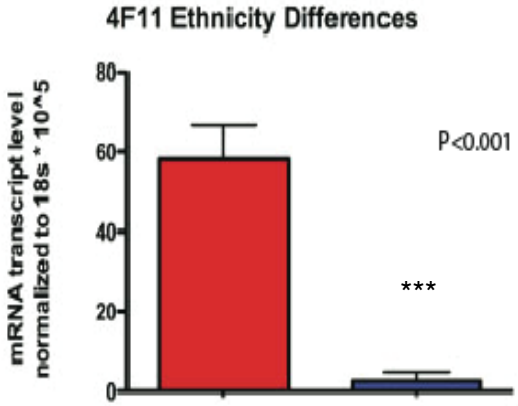
qRT-PCR transcript copy number comparison between African American and Caucasian samples to determine if ethnicity has an affect on CYP4F expression in the brain. CYP4F11 expression in African Americans is significantly higher than in their Caucasian counterparts. CYP4F2, CYP4F3B and CYP4F12 showed higher expression in Caucasian samples, but it was not statistically significant at the $P<0.05$ level. There also was no difference in CYP4F3A expression between the two ethnicities.


4F2 Ethnicity Differences
mRNA transcript level normalized to 18s * 10^5
15
10
5
0

4F3A Ethnicity Differences
mRNA transcript level normalized to 18s * 10^5
8
6
4
2
0


4F3B Ethnicity Differences
mRNA transcript level normalized to 18s * 10^5
30
20
10
0

4F12 Ethnicity Differences
mRNA transcript level normalized to 18s * 10^5
25
20
15
10
5
0


4F11 Ethnicity Differences
mRNA transcript level normalized to 18s * 10^5
80
60
40
20
0
P<0.001
***

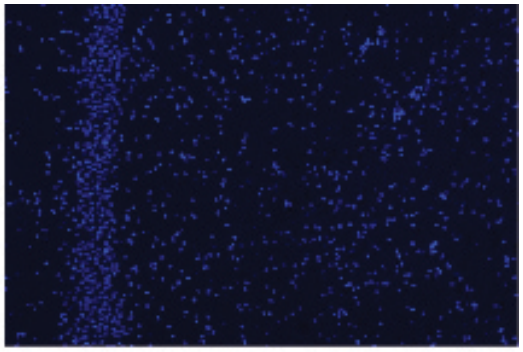
Cytochrome P450 4F11 is expressed selectively in both glial and neuronal cells

Immunofluorescent techniques were used to determine the expression of the protein in human brain samples. We used microtubule-associated protein 2 (MAP-2) to stain for neuronal cells, and glial fibrillary acidic protein (GFAP) to stain for glial cells, specifically astrocytes, in the brain (Figure 3.4 and Figure 3.5). CYP4F11 is being expressed in both glial and neuronal cells, but in specific cell types of each. The merged picture shows the overlap of CYP4F11 and either MAP-2 or GFAP. It also appears that the CYP4F11 is not present in the nuclei of these cells.

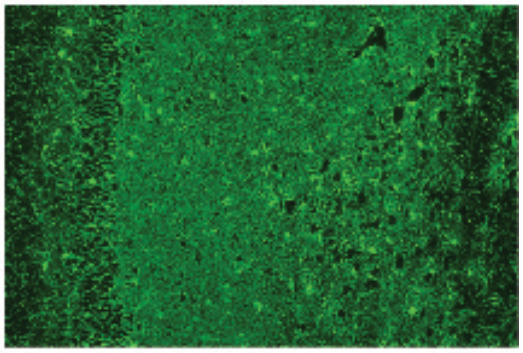
Figure 3.4. Localization of CYP4F11 Protein In Human Brain Glial Cells.

A) Immuno-fluorescent slide of human brain, DAPI staining of nuclei. B) GFAP (1:1000) in green staining glial cells C) CYP4F11 (1:100) in red. D) Merged picture showing overlap of GFAP and CYP4F11. There are also areas of no overlap representing CYP4F11 being expressed in other cells

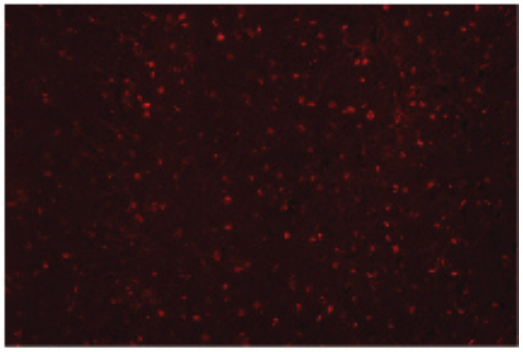
A.



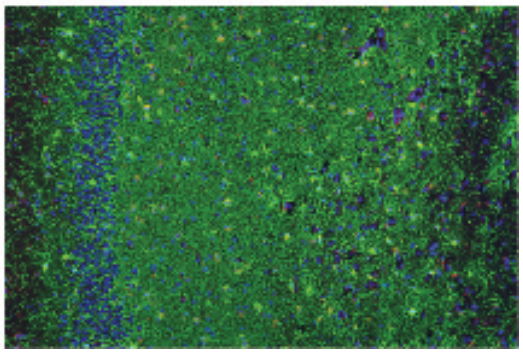
B.



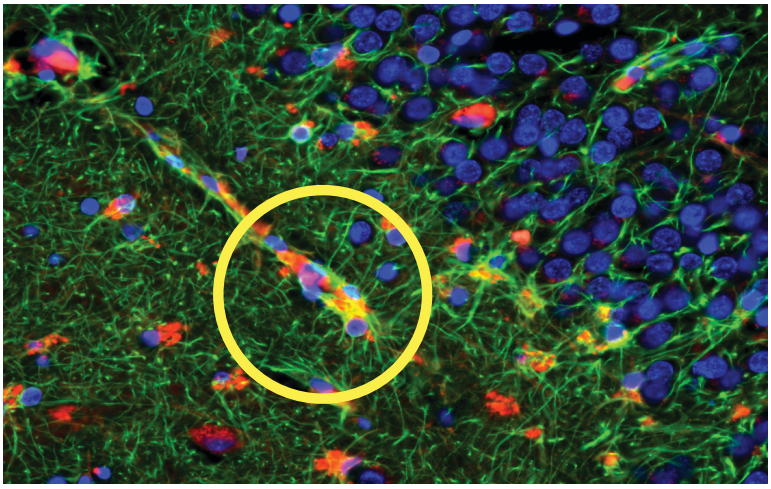
C.



D.



A.



B.

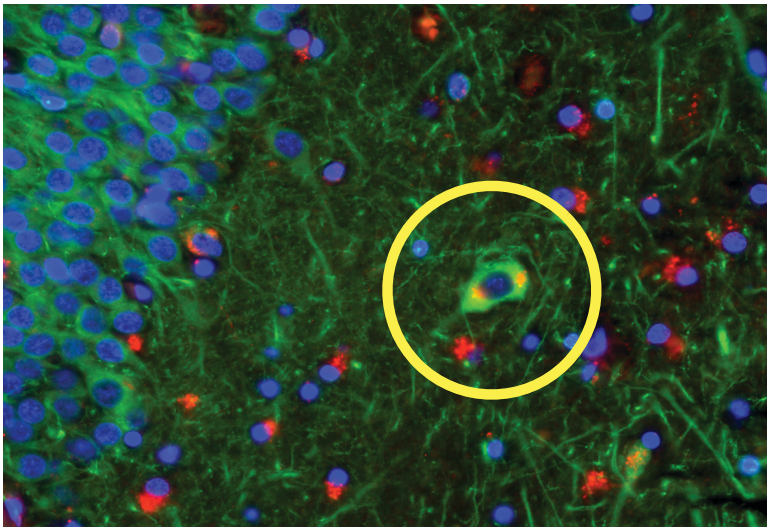


Figure 3.5. Localization of CYP4F11 Protein In Human Brain.

A) Immuno-fluorescent slide of human brain. CYP4F11 (1:100) in red and GFAP (1:1000) in green with DAPI staining of nuclei. Highlighted area shows that there is overlap of CYP4F11 and Glial Cells B) CYP4F11 (1:100) in red and MAP2 (1:1000) in green with DAPI staining of nuclei. Highlighted area shows that there is overlap of CYP4F11 and Neurons.

Discussion

Currently many cytochrome identification studies in the brain have shown the expression of the Cytochrome P450 families 1,2 and 3 in the brain [82-84]. However, recently there have been significant strides in identifying CYP4F expression in the brains of animals. These papers have focused on the importance of the endogenous compound metabolism and the pathways by which CYP4F metabolites may have on regulatory functions. There have been a few studies that identify human CYP4Fs in brain, but none that have taken a comprehensive look at the human CYP4F family expression [85-87]. In the current study we were able to show that the expression of CYP4Fs in the human orbital frontal cortex is dominated by CYP4F11. This finding is surprising because many CYP4Fs that are expressed in small quantities in the brain are expressed in large quantities in other tissues, as shown in Chapter 2 of this dissertation. However, what was not surprising was finding expression of CYP4F11. CYP4F11 was first identified and cloned from brain tissue and identified in other tissues as well, so its presence was expected [35]. The presence of a strong CYP4F11 expression provides further evidence that the brain may have evolved to express specific isoforms to perform multiple tasks. Kalsotra et al., reported that CYP4F11 was able to metabolize a great variety of pharmaceutical drugs including erythromycin, benzphetamine, imipramine and verapamil with acceptable turn-over rates [88]. However, the authors also show that although the turn-over rate for endogenous compounds such as leukotriene B_4 (LTB_4) is not as high as those of other CYP4F isoform

family members, CYP4F11 is still able to metabolize this compound and others such as the conversion of arachidonic acid (AA) to 20-HETE [88].

The presence of high CYP4F11 mRNA concentration in brain led us to investigate the localization of CYP4F11 protein in the brain. We found that CYP4F11 protein is located in both neuronal and glial cells. Since CYP4F11 is able to metabolize both endogenous compounds like AA and xenobiotics such as drugs, we believe this dual cell type expression is beneficial for the brain functions of signaling and detoxification. A majority of CYP enzymes have been reported to localize to neuronal cells [89, 90]. The localization of CYP4F11 in neuronal cells could prove to be important because the site of action for psychoactive drugs is in the neurons [91].

When determining the expression levels of each CYP4F isoform we found little to no expression of CYP4F2 and CYP4F3B in the brain, and as mentioned earlier in this dissertation these two enzymes metabolize AA into 20-HETE. As mentioned in the introduction of this chapter, 20-HETE is important in the neurovascular system of the brain and production of this compound would have to happen in the cells closest to the site of blood flow, such as the glial cells. The presence of CYP4F11 in the glial cells and the virtual absence of expression of CYP4F3B and CYP4F2 suggests a prominent physiological role for CYP4F11 in brain may be the conversion of AA into 20-HETE. Metea et al., were able to show that when the CYP4F ω-hydroxylation of AA is inhibited with HET0016 in glial cells, vasoconstriction is blocked [92].

We found that the expression of CYP4F11 protein may not be present in all neuronal and glial cells. Dutheil et al., recently published a review which reported when CYPs are expressed in the brain they are usually seen in specific cell types and are not ubiquitously expressed throughout brain regions [71]. Further research needs to be conducted with samples from a larger set of brain regions to define specific markers for the many different types of neuronal and glial cells in the brain.

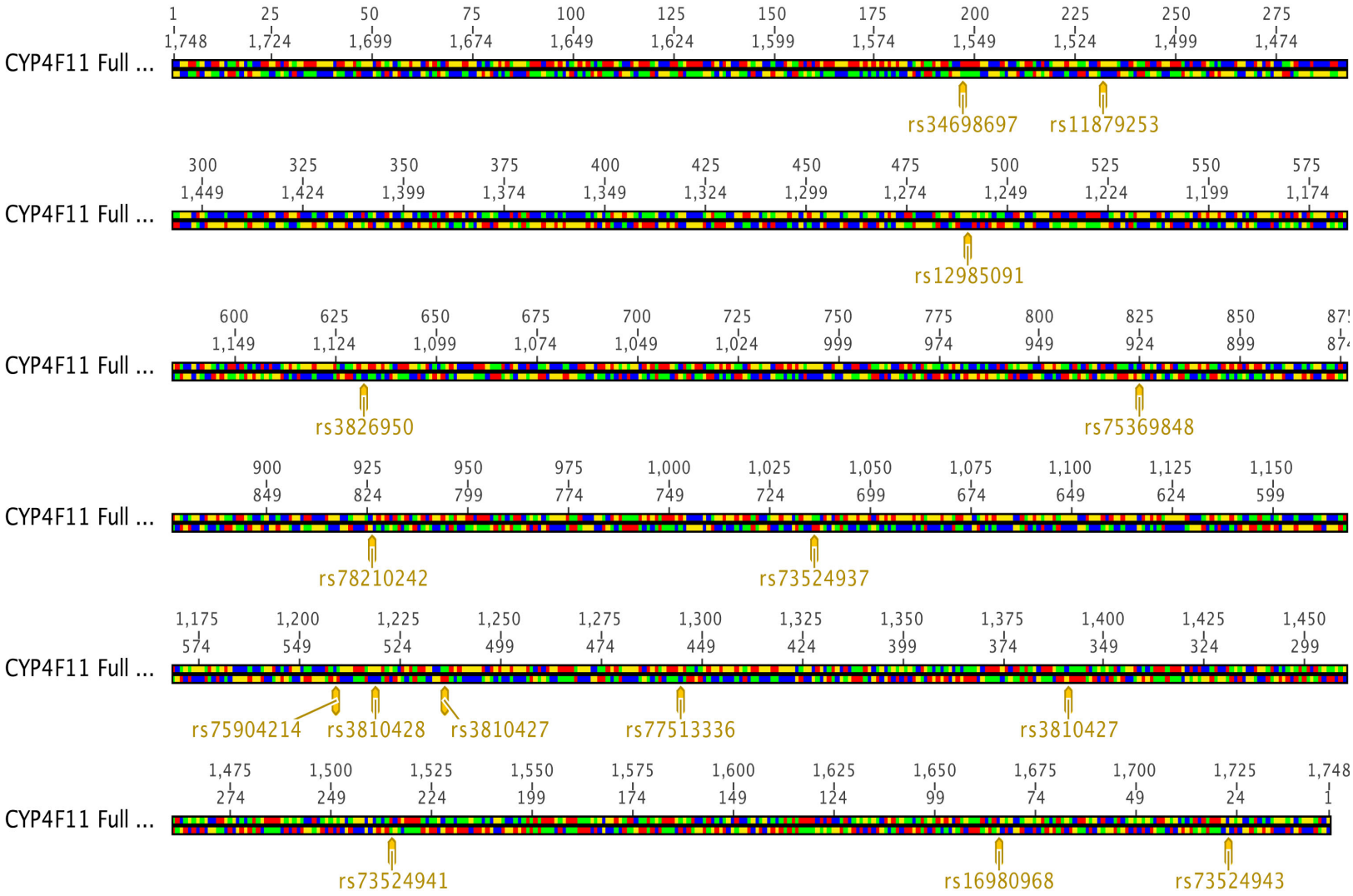
This current study also shows that gender plays no obvious role in the expression of CYP4F isoforms in the orbital frontal cortex. There have been many reports that provide evidence that gender can affect the expression of CYPs. Waxman et al., recently published a review of CYP families 1,2 and 3 showing how sex differences cause changes in expression of the CYPs [77]. In the future, with additional brain regions and larger sample sizes, a significant differences in expression between genders may emerge, but our study did not find any.

We did find that ethnicity-based differences in expression does occur between African Americans and Caucasians. Our data shows that African Americans express CYP4F11 at a significantly higher rate than Caucasians. The Ravindranath laboratory also found that in the Indian population CYP4F11 was expressed at a much higher level compared to the other CYP4F family members (personal communication). A large number of our sample group consisted of African American samples, so the addition of more Caucasian samples would help in identifying whether CYP4F11 can be expressed in a high quantity in this group. We examined the promoter region of CYP4F11 for single nucleotide polymorphisms (SNPs) Figure 3.6. Using HapMap (www.hapmap.org), we found

that a SNP labeled rs3810427(C/A) occurs at a rate of .868/.132 in African Americans while it is .496/.504 for most Caucasians while the other SNPs in Figure 3.6 were equal in both ethnicities. This means that the C nucleotide occurs 87% of the time in African Americans while in Caucasians this occurrence is only 50%. The SNP in this region could be in a critical promoter region binding site, or there also could be other SNPs in the gene that could be causing the difference in expression. This work is a preliminary attempt to understand the differences between the two ethnicities in CYP4F11 expression. Additional work is needed in this area to more fully understand any differences in expression. Other studies have also determined that ethnic differences play a role in the expression and function of many CYPs. Yamaori et al., for instance, reported that there was a difference in expression of CYP3A5 between Japanese and Caucasian subjects [81].

Our CYP4F expression work in human brain is a beginning for additional studies of CYP4Fs in the brain. As more work is done to identify the expression, localization and functional capabilities of CYPs in the brain is accomplished, there is a higher probability of identifying potential brain drug target opportunities and perhaps useful therapies.

Figure 3.6. 1.7kb 5'UTR of CYP4F11. The promoter region is marked with annotation of SNPs that occur in the population. SNPs were located using HapMap, www.hapmap.org. and sequence was analyzed using Geneious (BioMatters)


CYP4F11 Full ...
1 25 50 75 100 125 150 175 200 225 250 275
1,748 1,724 1,699 1,674 1,649 1,624 1,599 1,574 1,549 1,524 1,499 1,474
rs34698697
rs11879253
CYP4F11 Full ...
300 325 350 375 400 425 450 475 500 525 550 575
1,449 1,424 1,399 1,374 1,349 1,324 1,299 1,274 1,249 1,224 1,199 1,174
rs12985091
CYP4F11 Full ...
600 625 650 675 700 725 750 775 800 825 850
1,149 1,124 1,099 1,074 1,049 1,024 999 974 949 924 899
rs3826950
rs75369848
CYP4F11 Full ...
900 925 950 975 1,000 1,025 1,050 1,075 1,100 1,125 1,150
849 824 799 774 749 724 699 674 649 624 599
rs78210242
rs73524937
CYP4F11 Full ...
1,175 1,200 1,225 1,250 1,275 1,300 1,325 1,350 1,375 1,400 1,425 1,450
574 549 524 499 474 449 424 399 374 349 324 299
rs75904214
rs3810428
rs3810427
rs77513336
rs3810427
CYP4F11 Full ...
1,475 1,500 1,525 1,550 1,575 1,600 1,625 1,650 1,675 1,700 1,725 1,748
274 249 224 199 174 149 124 99 74 49 24 1
rs73524941
rs16980968
rs73524943

Chapter Four: Regulation of Cytochrome P450 4F11 by Nuclear Transcription Factor-Kappa B

Abstract

The mechanisms that regulate CYP4F genes are currently being studied in a number of laboratories, but the specific mechanisms for the regulation of these genes are not yet fully understood. This study shows that nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) can inhibit CYP4F11 expression in human liver carcinoma cell line (HepG2). Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine has been shown to activate NF-κB signaling while also making active the c-Jun N-terminal Kinases (JNK) signaling pathway. Previous studies have reported that JNK signaling can up-regulate CYP4F11 expression, and in this study we have determined that in the presence of TNF-α and the specific NF-κB translocation inhibitor IMD-0354 there is a greater increase in CYP4F11 expression, indicating that NF-κB may be playing an inhibitory role. Furthermore, NF-κB stimulation by over-expression of Mitogen-activated protein/ ERK Kinase Kinase (MEKK) inhibited CYP4F11 promoter expression. We were also able to rescue CYP4F11 promoter inhibition in the presence of TNF-α when p65, a NF-κB protein, was knocked down. Thus, the CYP4F11 gene is negatively regulated by NF-κB signaling pathways.

Background

The release of several cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-1b, and IL-6 from activated immune cells in patients with a disease with an inflammatory component, or infection occur as part of the activation of systemic host defense mechanisms. This defense mechanism and the release of these cytokines usually results in the down-regulation of many isoforms of cytochrome P450 (CYP) [93-98]. This is important due to the known responsibilities of the CYP systems in the metabolism of drugs used in treatment of such conditions as well as steroids, lipid soluble vitamins, prostaglandins, leukotrienes. If there is a disruption in the balance of these enzymes to metabolize their substrates there could be an alteration in the production or elimination of these substances and an interruption in the balance between the detoxification and metabolic bioactivation in the body.

One of the prominent cytokines, TNF-α, is known to be released during many types of infections and inflammatory diseases [95], and in our current study we used this cytokine to define the effects its presence has on the regulation of cytochrome P450 4F11, an isoform of the 4F family that has a substrate profile that includes not only endogenous compounds but also xenobiotics specifically drugs [88]. TNF-α stimulation causes an activation of two signal transduction pathways the c-Jun N-terminal Kinases (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways [99-101]. Our previous studies have examined the role JNK and retinoic acids play in the regulation of CYP4F11 and have found that JNK stimulation causes an increase in CYP4F11 mRNA while

retinoids cause down-regulation of CYP4F11 mRNA [102]. However, we did not examine the effects of NF-κB on CYP4F11 expression during TNF-α stimulation.

To understand at a deeper level the regulation of CYP4F11 expression during inflammation we show in this study that TNF-α and MEKK over-expression can down-regulate CYP4F11 expression in NF-κB dependent manner. Our results suggest that there is an intricate regulatory system for control of expression of CYP4F11 during conditions wherein inflammatory modulators are released.

Materials and Methods

Chemicals

N-[3,5-Bis(trifluoromethyl)phenyl]-5-chloro-2-hydroxybenzamide (IMD-0354) was obtained from Sigma-Aldrich (St. Louis, MO). TNFα was a gift from Dr. Jianping Jin (University of Texas-Houston). IL-1 were bought from Invitrogen Inc. (Carlsbad, CA). Polyclonal anti-CYP4F11 antibody was provided by Proteintech Group, Inc. (Chicago, IL). Rel B and p65 antibodies were bought from Cell Signaling (Danvers, MA).

Cell Culture

HepG2 cell line was obtained from American Type Culture Collection (Manassas, VA). Cells were grown at 37^{o}C in a humidified incubator with 5% CO_2 in Minimum Essential Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), L-glutamine and Pen/strep antibiotics.

Protein Isolation and Western Blot Analyses

Protein isolation and western blot analyses for cells in culture were accomplished using the protocol reported by Wang et al., 2010 [102]. Briefly, Cells were washed two times with ice cold phosphate-buffered saline and then quickly detached by gently scraping the plate. Collected cells were spun down at 4500rpm for 2min at 4^{o}C, in a microcentrifudge and stored at -80^{o}C. Lysates were then prepared using 50mM Tris-HCl buffer, pH6.8, with 2% SDS. Cell pellets were dissolved in the lysate buffer then boiled at 95^{o}C for 5 minutes. The cell lysate was then cleared by centrifugation at 13,000 rpm for 5 minutes in a microcentrifudge. Protein concentrations of the clear supernatant fraction were measured using the bicinchoninic acid assay kit (Pierece, Rockford, IL) and subjected to Western blot analysis.

Protein samples were boiled in Laemmli buffer and resolved electrophoretically on 4 - 20% gradient Tris-glycine-SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to polyvinylidene fluoride membranes (wet transfer apparatus; Bio-Rad Laboratories). Membranes were blocked with 5% dried non-fat milk in Tris Buffer with .05% Tween for 1 hour. Membranes were incubated overnight with antibodies raised against CYP4F11 (1:1000 dilution), Rel B (1:1000), p65 (1:1000), or GAPDH (1:10,000). The membrane was then washed and incubated at room temperature with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) for 1 hour. Immunoreactivty was visualized using a horseradish peroxidase chemiluminescense system (Pierce).

Quantitative Real-Time Polymerase Chain Reaction

Cells were rinsed with phosphate-buffered saline (PBS), and total RNA was isolated using TRIzol reagent (Invitrogen). DNase I (Invitrogen) was used to alleviate DNA contamination. Aliquots (100 ng) of total RNA were reverse-transcribed by SuperScript II Reverse Transcriptase (Invitrogen) in triplicate, including a reverse transcription blank to evaluate presence of contaminating genomic DNA. Amplification was performed using Taq DNA Polymerase (Invitrogen) with an ABI Prism 7700 (Applied Biosystems, Foster City, CA) at 95^{o}C for 1min, followed by 40 cycles at 95^{o}C for 12s and 60^{o}C for 30s. *CYP4F11* mRNA levels were measured using standard curves generated by plotting Ct versus the log of the amount of purified amplicon for *CYP4F11* (custom synthesis by Invitrogen) (200ag - 2pg). Abundance of human 18S RNA was used as an internal control. Primers and fluorescent probe sequences for CYP4F11 and 18S RNA are reported in Table 4.1.

Plasmids

pGL3-CYP4F11 plasmid mutants were constructed using human genomic DNA as a template for PCR. The primer pairs for each mutant are listed in Table 5.2. The products were all 3' promoter regions of the CYP4F11 (GenBank accession no. NG_0008335). The PCR products were then cloned into HindIII- and NheI- digested linear pGL3 Basic lucifearse vector (Promega, Madison, WI) using the infusion Advantage PCR Cloning Kit (Clontech, Mountain View, CA).

Transfection and Luciferase Assays

Cells were transfected using Fugene HD Reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. Cells were seeded onto 24 well plates (5 x 10^4 cells/well). 24 hours after seeding, cells were transfected at a recommended Reagent:DNA ratio of 4.5:1 with 0.5 *μg* DNA/well including .03 *μg* of phRL-SV40 (Promega). Total amount of DNA was maintained at a constant concentration by adding empty pcDNA3 vector when appropriate. After 24 h of transfection, TNF-α (10 ng/ml) or IMD-0354 (1 ng/ml) treatment was given for 24 h.

RNAi

Double-stranded, siRNAs targeting p65 were designed and synthesized by Sigma-Aldrich (St. Louis, MO) 5'GACAUUGAGGUGUAUUUCA3', 5'UGAAAUACACCUCAAUGUC3', and reverse transfected into HepG2 cells using Lipofectamine RNAiMAX (Invitrogen). In short, 24 well plates were seeded (5 x 10^4 cells/well) in the presence of 6 (pmol) and 1*μl* of Lipofectamine RNAiMAX in 100*μl* of Opti-Mem I Serum (Invitrogen). Total media volume was 600*μ*l for a final RNAi duplex concentration of 10 nM.

Chromatin immunoprecipitation (ChIP) analysis

Binding of p65 transcription factor to the human CYP4F11 promoter region was determined by ChIP assay according to manufacturer's protocol (Active Motif, Carlsbad, CA). HepG2 cells after either 1 or 24 h treatment with TNF-α (10ng/ml) were fixed using 1% formaldehyde in modified Eagle's medium for 10 min at 37^{o}C. The cells were then washed 1x in cold PBS, then treated with 125 mM glycine to

terminate the cross-linking reaction. The cells were washed again with ice cold PBS, and collected in PBS containing 1mM phenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail (Active Motif, Carlsbad, CA). The cells were lysed and sonicated to shear chromatin. 15 μg of chromatin was then incubated overnight at 4^{o}C with antibody directed against p65. PCR was used to amplify the 222bp region upstream of the start site of CYP4F11 from the purified DNA-protein immune complexes. PCR products were run on 2% agarose gel and visualized after ethidum bromide staining. Controls for the assay were performed using IgG and input template DNA.

Statistical Analysis

Data are presented as mean ± S.E.M. One-way analysis of variance followed by Dunnett's multiple comparison test was used for the statistical analysis. Statistical differences were considered significant if $P < 0.05$.

Database Sequence Analysis

The TRANSFAC database (BIOBASE, Beverly, MA) was searched using AliBaba 2.1 (BIOBASE) for putative NFκ-B binding sites on the 5' flanking sequence (2000 base pairs from the ATG start codon) of *CYP4F11* gene.

Results

NF-κB inhibition increases expression of CYP4F11

Our lab recently published that CYP4F11 expression is increased upon stimulation with TNF-α in Keratinocyte HaCaT cells [102]. TNF-α activates two pathways, one being the JNK pathway which we have reported can up-regulate CYP4F11 through AP-1 binding site, and the NF-κB signaling pathway. It was our intention to understand what role this transcription factor played in regulation of CYP4F11. We first determined that 24-hour treatment of TNF-α (10ng/ml) caused an up-regulation of CYP4F11 mRNA in HepG2 cells measured by quantitative real-time polymerase chain reaction (qRT-PCR). To understand the role NF-κB may have on the expression of CYP4F11, we inhibited the ability of NF-κB to translocate to the nucleus from the cytoplasm using the chemical inhibitor IMD-0354 (1ng/ml). HepG2 cells were co-treated with both the inhibitor and TNF-α (10ng/ml) (Figure 4.1). There was a significant increase in the expression of CYP4F11 transcripts as compared to control vehicle. Realizing that NF-κB played a role in CYP4F11 expression after observing an up-regulation of the gene when translocation of NF-κB to the nucleus was inhibited, we looked 2000bp upstream of the start site of the gene in TRANSFAC database using the program AliBaba 2.1 for predictions of transcription factor binding sites (Figure 4.2). The website predicted 5 different NF-κB binding sites in the promoter region of CYP4F11.

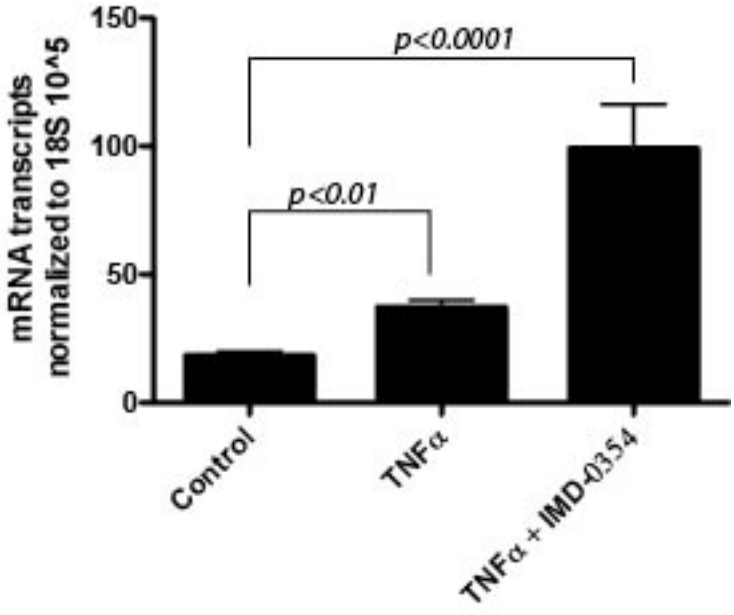


Figure 4.1. NF-κB inhibition increases expression of CYP4F11. Up-regulation of CYP4F11 transcripts by inhibition of NF-κB. HepG2 cells were treated with 10ng/ml of TNF + or - IMD-0354 (1ng/ml) NF-κB inhibitor for 24 hours. Cells treated with 0.1% DMSO were used as the vehicle control. Expression of CYP4F11 was quantitated by qRT-PCR. Each data point represents n=6

Figure 4.2. NF-κB binding site predictions. 2000bp upstream of ATG start site for *CYP4F11,* predictions calculated through AliBaba 2.1.

CYP4F11 promoter activity is downregulated after HepG2 activation by TNF-α and MEKK overexpression.

To determine the TNF-α regulatory region in the *CYP4F11* promoter, we cloned the 1.7kb region upstream of *CYP4F11* exon-2 transcription initiation site into pGL3 basic luciferase vector. We first determined the basal promoter activity, then activity after treatment with TNF-α and MEKK overexpression in HepG2 cells. As seen in Figure 4.3A, MEKK and TNF-α treatment of the transfected cells resulted in a 70-75% reduction in *CYP4F11* promoter activity. TNF-α and MEKK treatment up-regulated control NF-κB luciferase reporter vector activity (Figure 4.3B) but had no effect on pGL3 basic luciferase control vectors Figure 5.C. This suggests that even though TNF-α causes an overall up-regulation of *CYP4F11* transcripts as seen previously in Figure 4.1, the promoter inhibition is accomplished through NF-κB activation. These data are confirmed through the over-expression of MEKK which causes a constitutively active NF-κB protein that also down-regulates CYP4F11 promoter activity.

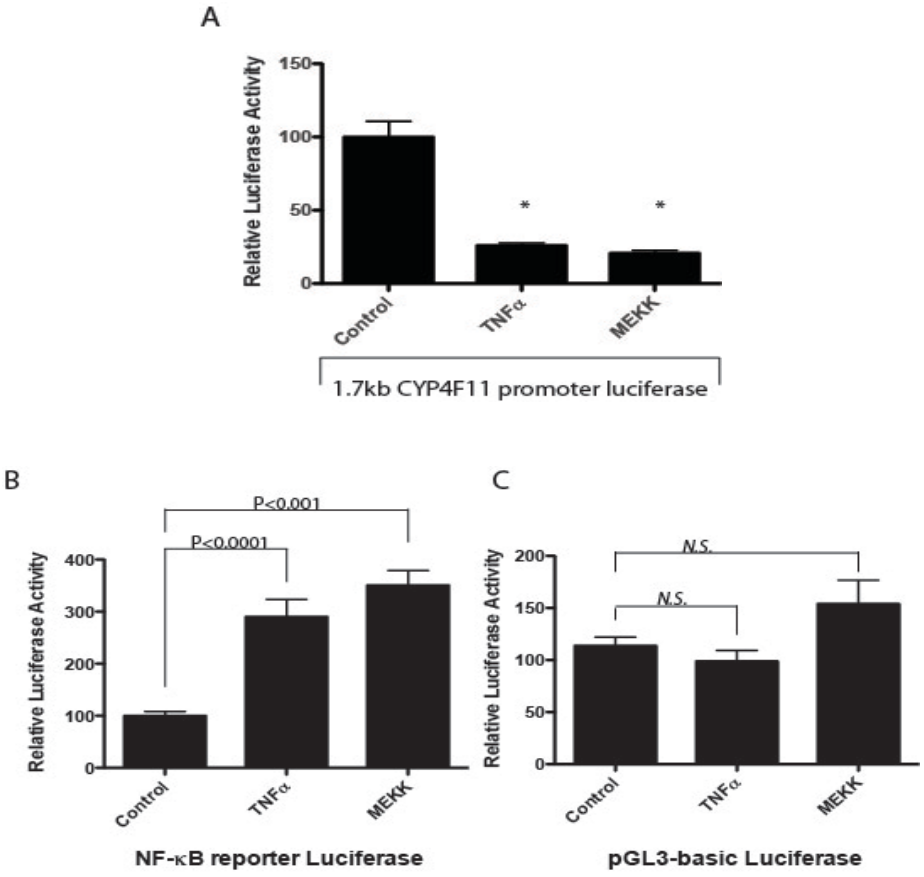


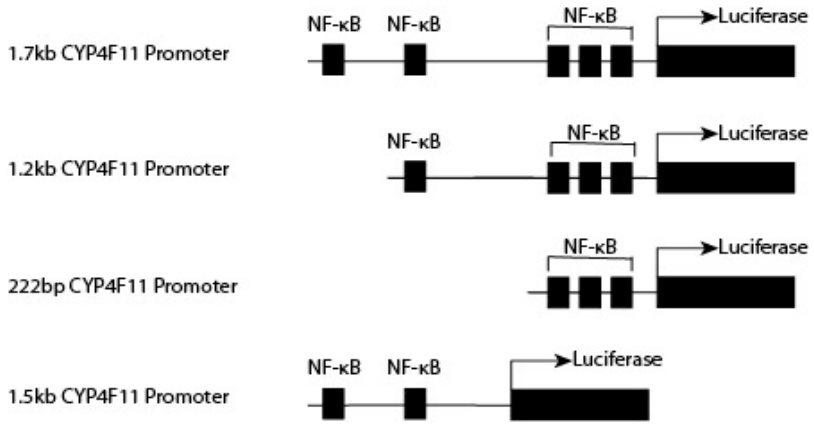
Figure 4.3. TNF-α and MEKK suppresses CYP4F11 promoter activity. HepG2 cells were transfected with 1.7kb *CYP4F11* promoter-pGL3 basic luciferase (A) or NF-κB luciferase (B) or pGL3-basic luciferase (C) reporter vector. 24 hours post-transfection, the cells were treated with TNF-α (10 ng/ml) for 24 hours and luciferase activity was measured. Measuring *Renilla* luciferase activity of co-transfected phRL-SV40 normalized the transfection efficiencies.

NF-κB responsive region is present in the first 200bp of CYP4F11 promoter

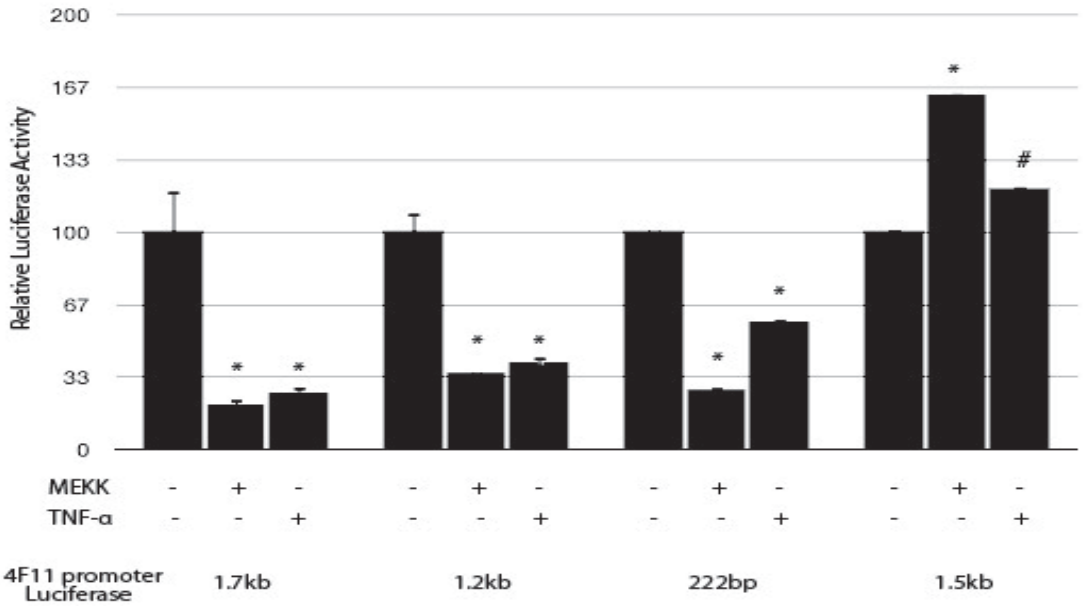
The prediction of five NF-κB binding sites by AliBaba 2.1 enabled us to create three deletion mutants to determine the functional importance of these predicted binding sites (Figure 4.4A) Each construct deletes a predicted NF-κB binding site. The last 1.5kb mutant construct deleted 3 predicted NF-κB binding sites that were within 100bp of each other. HepG2 cells were transiently transfected with all the 5' deletion constructs with or without MEKK expression plasmid and treated with or without TNF-α. All the 5'-deletion constructs of the CYP4F11 promoter were down regulated by TNF-α and MEKK except the 1.5kb bp CYP4F11 promoter (Figure 4.4B). This result suggests that the NF-κB responsive region should be within the first 200bp of CYP4F11 promoter. A chromatin immunopercipitation assay was run on the first 222bp of CYP4F11 promoter region to see whether the p65 transcription factor was binding to the promoter region to cause an inhibition of the promoter. Figure 4.5 shows that the protein does bind to the region, and the relative amount of protein binding the region is TNF-α activation time-dependent. The time-dependency of activation of NF-κB and JNK have been reported by many labs [101, 103].

Figure 4.4. NF-κB responsive region present in the first 200bp of CYP4F11 promoter. (A) Deletion constructs of CYP4F11 promoter (B) HepG2 cells were transiently co-transfected with 4 different 5'-deletion constructs of CYP4F11 promoter luciferase reporter with or without MEKK-pcDNA3 and phRL-SV40 to normalize the transfection efficiencies. TNF-α (10 ng/ml) treatment was initiated 24 hours post-transfection for 24 hours. Luciferase activity was measured 48 hours after transfection.

A


NF-κB
NF-κB
NF-κB
Luciferase
1.7kb CYP4F11 Promoter
NF-κB
NF-κB
Luciferase
1.2kb CYP4F11 Promoter
NF-κB
Luciferase
222bp CYP4F11 Promoter
NF-κB
NF-κB
Luciferase
1.5kb CYP4F11 Promoter

B


Relative Luciferase Activity
200
167
133
100
67
33
0
MEKK
TNF-α
4F11 promoter Luciferase
1.7kb
1.2kb
222bp
1.5kb

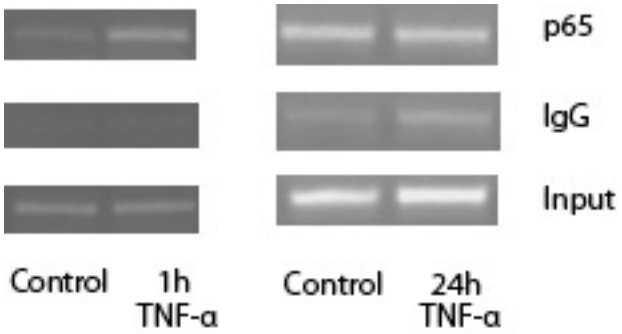
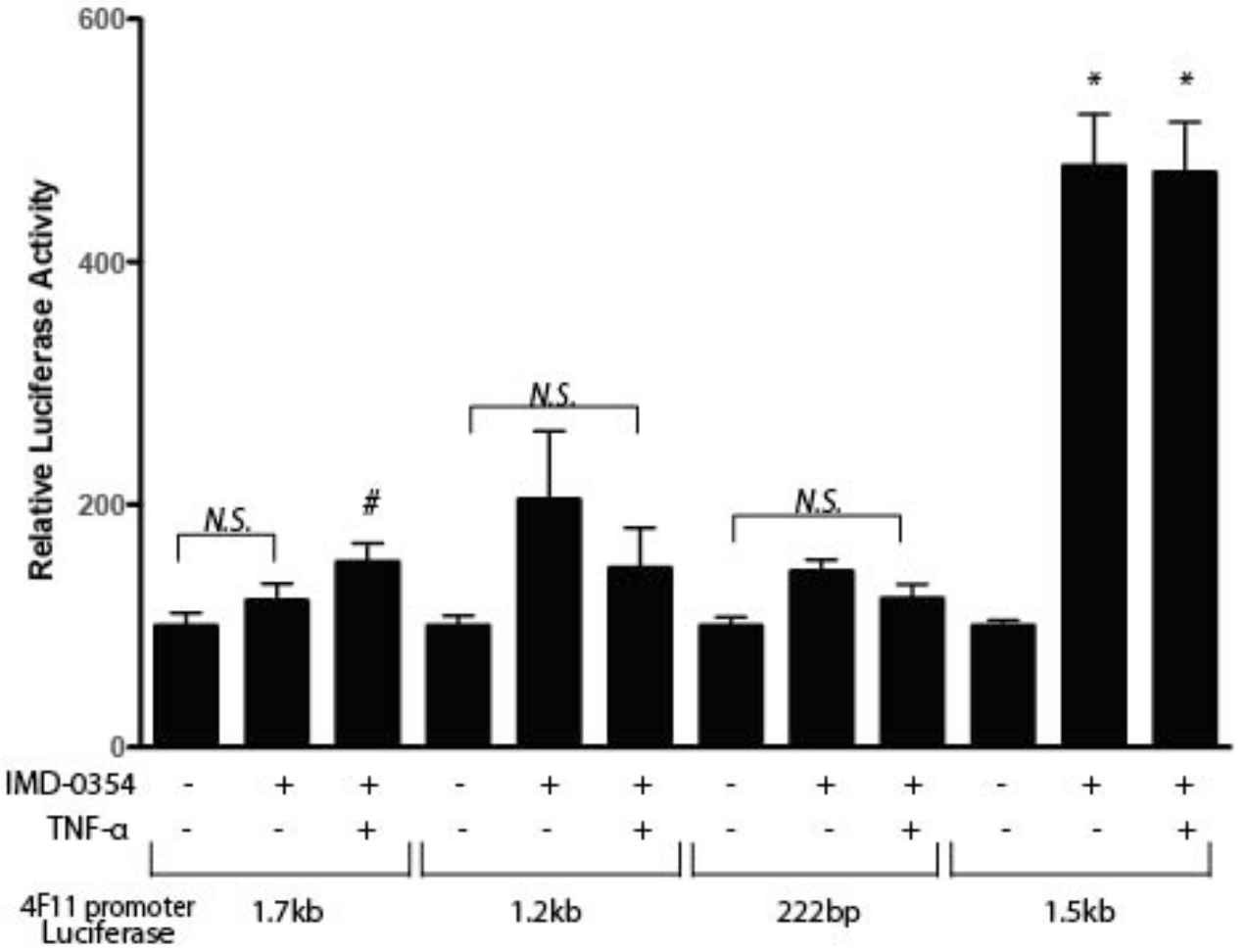


Figure 4.5. NF-κB binds to first 200bp region of CYP4F11 promoter. ChIP assays performed in HepG2 cells that had been treated with TNF-α (10 ng/ml) or vehicle control for 1 hour and 24 hours. The pull down of p65 is seen in the 200bp amplicon region in both 1 and 24 hours.

NF-κB is required for CYP4F11 down-regulation

The next goal was to determine the importance of NF-κB in the down-regulation of *CYP4F11* in response to TNF-α. We achieved this by using the IKKα phosphorylation inhibitor IMD-0354. This drug inhibits the translocation of NF-κB to the nucleus, thus preventing any regulatory controls NF-κB exerts on its response genes. HepG2 cells were transiently transfected with each CYP4F11 deletion luciferase promoter construct, then co-treated with TNF-α (10 ng/ml and IMD-0354 (1 ng/ml). As shown in Figure 4.6, CYP4F11 promoter activity is not down-regulated by TNF-α in the presence of IMD-0354. To make sure there were no drug interactions that were affecting the results, the experiment was repeated in HepG2 cells in the presence of siRNA that knocks down p65 thus preventing expression. Figure 4.7 shows that after p65 knockdown and in the presence of TNF-α or over-expression of MEKK down-regulation of CYP4F11 does not occur. These data clearly suggest that CYP4F11 down-regulation requires p65 or NF-κB.

Figure 4.6. IMD-0354 inhibition of NF-κB releases inhibition of CYP4F11 transcripts. HepG2 cells were transiently transfected with CYP4F11 deletion promoter constructs 24 hours after being plated. Cells were then co-treated with TNF-α and IMD-0354 (NF-κB translocation inhibitor) 24 hours post transfection for 24 hours and then luciferase activity was measured. Measuring *Renilla* luciferase activity of co-transfected phRL-SV40 normalized the transfection efficiencies.


600
400
200
0
Relative Luciferase Activity
N.S.
#
N.S.
N.S.
*
*
IMD-0354 - + + - + + - + + - + +
TNF-α - - + - - + - - + - - +
4F11 promoter Luciferase
1.7kb
1.2kb
222bp
1.5kb

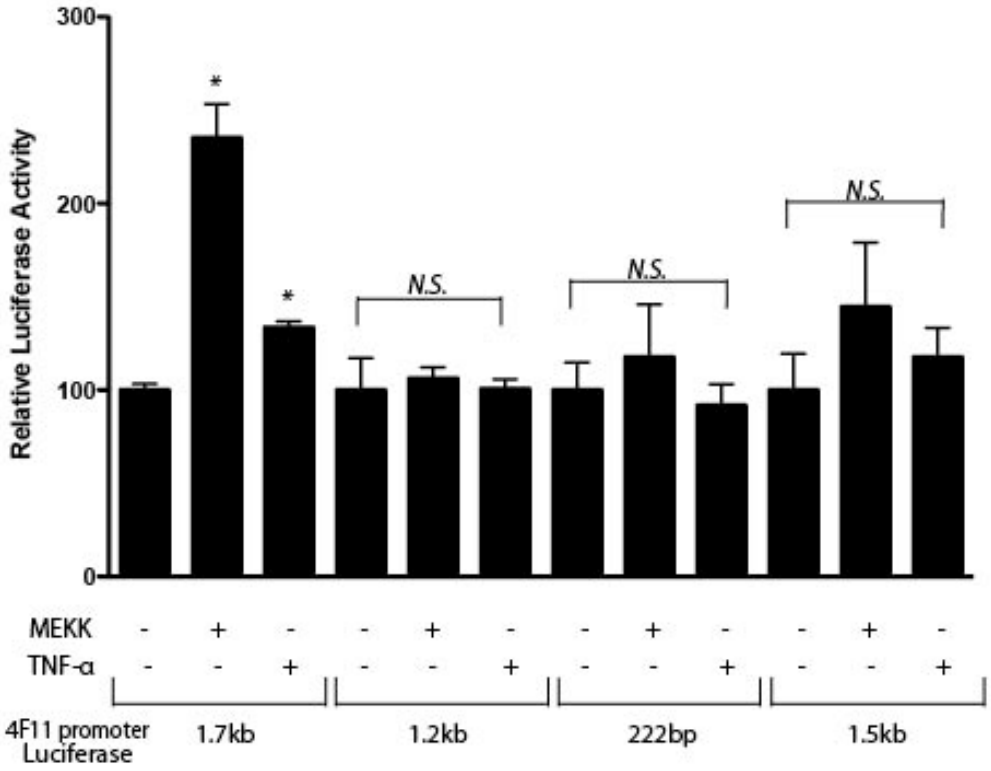


Figure 4.7. TNF-α and MEKK mediated down-regulation of CYP4F11 promoter is NF-κB dependent: HepG2 cells were reverse transfected with siRNA against p65 with lipofectamine RNAiMAX. 24 hours post transfection, HepG2 cells were transiently transfected with CYP4F11 luciferase deletion promoter constructs. Cells were then treated with TNF-α 24 hours post luciferase transfection for 24 hours and then luciferase activity was measured. Measuring *Renilla* luciferase activity of co-transfected phRL-SV40 normalized the transfection efficiencies.

Discussion

The ability for cytokines, prostaglandins and other inflammatory mediators to alter the expression of many different CYPs has been the subject of many different studies [61, 97, 104, 105]. The importance of this work stems from the effects that changes in CYP levels have on the metabolism of many drugs, on the homeostasis of steroid hormones and on the ability of CYPs to detoxify xenobiotics. The effects of inflammation on CYP expression are varied, but the predominant effect is that inflammatory cytokines suppress the gene expression of most CYPs. In the current study we have presented a novel regulatory role for CYP4F11 expression during an inflammatory response. We have shown that activation of the NF-κB pathway by TNF-α stimulation or over-expression of MEKK causes down-regulation of CYP4F11 transcripts. However, what makes this unique is that the inhibition of native CYP4F11 is not seen in the presence of TNF-α after 24 hours (Figure 4.1) as was seen in this study and in a previous study [102]. We believe this is due to the signal transduction properties of TNF-α. It is believed that the c-Jun N-terminal Kinases (JNK) pathway and the NF-κB pathways are both stimulated in the presence of TNF-α; however, they are competing forces [106]. The up-regulation of CYP4F11 mRNA in the presence of TNF-α is due to the stimulation of the JNK pathway [102], however this is time dependent and does not reach a level of significance until 24 hours after treatment as observed in our laboratory. This was confirmed in the ChIP analysis where at 1 hour there was a large increase in p65 bound CYP4F11 transcripts that remained constant at 24 hours.

The inhibition seen in CYP4F11 promoter constructs is driven mainly by NF-κB activation. We were able to show this in various experiments including inhibiting translocation of NF-κB to the nucleus and then treating HepG2 cells with TNF-α (Figure 4.1). This resulted in a large increase of transcripts leading us to believe that although JNK is a strong stimulator of CYP4F11 expression, NF-κB may be playing an inhibitory role. After determining that NF-κB inhibition caused a decrease in promoter activity in our constructs (Figure 4.4) we then utilized chemical inhibition (Figure 4.6) and protein knockdown (Figure 4.7) of NF-κB and determined the effects on the promoter activity. We found that in those cases there was either control level expression or over-expression of CYP4F11 gene. This led us to believe that the down-regulation of CYP4F11 is NF-κB dependent. The cases of over-expression can be explained by the activation of the areas in the promoter region that have AP-1 binding sites which would create higher levels of expression of CYP4F11 in the presence of JNK activation. This mechanism was described in a recent paper published by our lab in Drug Metabolism and Disposition 2010 [102]. In short, JNK activation causes activation of AP-1 protein which regulates expression through the AP1 binding site on a gene's promoter. In the 1.7kb CYP4F11 promoter construct there are five AP-1 binding sites positioned throughout the promoter region and when TNF-α is present JNK signaling can still occur and elicit regulation of the CYP4F11 promoter construct. Overall, this finding is just one piece of a very complicated mix of regulatory networks that lead to the regulation of CYP4F11, and with each finding the pieces of the puzzle are beginning to fit. This is pictorially represented in Figure 4.8. Overall, we believe

that this regulation of CYP4F11 may be an important mechanism of compensation in cells. As most CYPs are down regulated during the inflammatory response it is unique that CYP4F11 is up-regulated. CYP4F11 has the ability to compensate for the metabolism profiles of CYP4F2 and CYP4F3A/B as these enzymes are down-regulated during an inflammatory response. However this theory needs to be tested further with functional studies that can show the effects of inflammatory response on activity. This, in addition to regulatory sites found for CYP4F11, may help investigators develop drug therapies to one or several regulatory sites to modulate pathological inflammation.

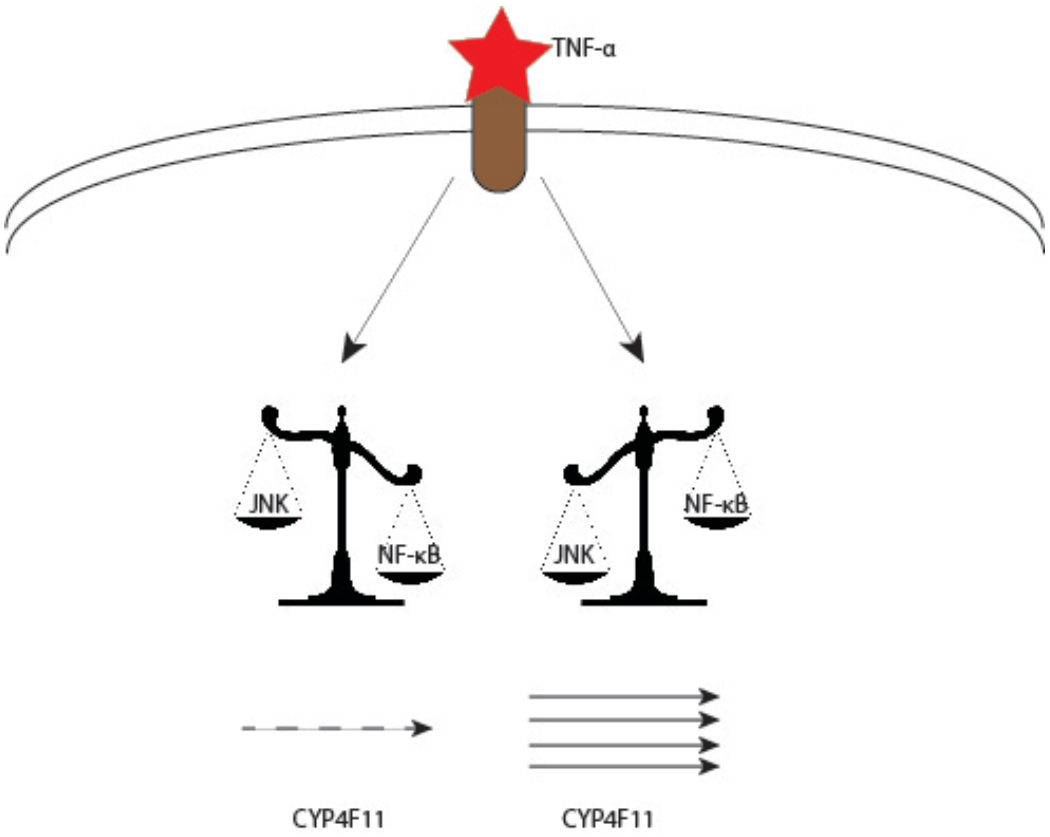


Figure 4.8. Summary of TNF-α Regulation of CYP4F11 Expression. Diagram of regulation of CYP4F11 expression. TNF-α activates pathways for JNK and NF-κB, these two pathways although both activated are able to regulate genes that oppose each other. What we have found is that the longer TNF-α is present and activating its receptor the more the JNK pathway is activated, this activation up-regulates CYP4F11 expression, however other signaling that can activate NF-κB would cause an inhibition of CYP4F11.

Chapter Five: Conclusions, Significance and Future Directions

Localization of CYP4Fs

Although work on the Cytochrome P450 4F family began in the 1990's there, is still more work that needs to be done to understand fully all the characteristics of this family. This current work in identifying the mRNA level of expression of the CYP4F family in different human tissues is novel in itself and is an important step in translating what we have learned in the animal models to human studies. While localizing and determining the relative quantities of the CYP4Fs in the liver, lung, spleen, kidney, heart and brain are very important, finding that these enzyme profiles change in the presence of inflammatory mediators helps in understanding regulation of these enzymes. This is important because it is the first time its been shown that human patient samples change CYP4F expression levels in a disease state. It also demonstrates the importance of animal model testing, where studies have shown that injury causes significant changes in the CYP4F expression profile.

It is important to next examine the protein levels of each isoform in these tissues. This has currently been a problem because of 1) the high homology between the CYP4F family which leads to 2) lack of specific antibodies for each isoform. This approach will show how the mRNA expression level relates to the protein functional expression in tissues. Another approach that should be pursued is identifying a specific inflammatory disease and obtain tissue samples from those patients in order to compare them to a specified control to determine the exact effects a known inflammatory disease model has on CYP4F expression and what functionality gain or loss is associated with it.

CYP4F11 expression in the brain

This research project identified the expression profile of the CYP4F family in the brain, and found hat CYP4F11 was expressed at high quantities. Knowing that CYP4F11 has a diverse substance specificity that ranges from xenobiotics to endogenous compounds, makes this finding even more relevant and important. It is important to note that the orbital frontal cortex is responsible for integrating emotion with behavior and is the site of a large number of neurotransmitter signaling processes. With the knowledge that 1) CYP4F11 is expressed at high levels in this region and 2) that the expression is present in both the neurons, site of neurotransmitter action, and glial cells, site or neuroinflammatory control, there is the possibility that the enzyme may be a potential drug target for different mental disease states affecting this region of the brain.

Future work studies should include additional brain regions. This study focused on the orbital frontal cortex, but the addition of other regions is needed because of the specificity that is seen in other CYPs expressed in the brain. Functional studies of CYP4F11 in the frontal cortex would also be beneficial to determine how this enzyme can function as a drug target.

NF-κB inhibits CYP4F11 expression

Work in our laboratory has focused on the regulation of the CYP4Fs during an inflammatory response. We have focused on this because of the role CYP4Fs play in decreasing inflammatory mediators such as leukotrienes and prostaglandins. This current study determined that CYP4F11 is negatively

regulated by the activation of NF-κB. The importance of this finding is that while other research has shown that CYP4F11 is up-regulated by TNF-α stimulation this current research determined that if NF-κB is blocked and cells are stimulated by TNF-α there is a three fold increase of expression. As more work is done on the role CYP4F11 has during an inflammatory response this information will be of use in determining the link between inflammation, CYP4F11 and cell metabolism function.

Conclusion

This research project has many parts that when placed together create a picture of how CYP4Fs are expressed and how inflammatory mediators especially TNF-α, play an important role in the regulation of this family of enzymes. We believe that the expression of CYP4F2 and CYP4F3B at high levels across a diverse array of tissues helps to create homeostasis in the body by regulating LTB_4 levels and 20-HETE levels in their specific organs. As inflammatory response signals infiltrate the tissue they are able to regulate the expression of these enzymes so that the normal host defense mechanism can take place. In the example of the liver presented in Chapter 2, inflammation increased CYP4F11 expression and the presence of CYP4F3B, but at the same time it caused a decrease in CYP4F2 expression. These regulatory functions are necessary both for the normal host defense process and then the subsequent return to normal functional state. The reduction in CYP4F2 helps to keep LTB_4 present at the site of infection or injury so that neutrophils can be recruited to area. The increase in

CYP4F11 and CYP4F3B helps to increase the levels of 20-HETE which has also been associated with the presence of inflammation [107]. As all of this is taking place during the pro-inflammatory state, the switch to the anti-inflammatory stage leads to a time dependent increase in CYP4F2, which provides greater LTB_4 metabolism while also decreasing the presence of CYP4F3B and CYP4F11 expression. This hypothesis of regulatory controls still needs further elaboration, but the work done in this current research project has added new pieces to the puzzle.

Vita

Jordan Bell was born in Houston Texas to Charlean Jordan and Ivy Bell. He has two brothers, one older and one younger Ivy and Charles respectively. He grew up in the Cypress Fairbanks school district, then was accepted to the University of Rochester to pursue a B.S. degree in Neuroscience. His first brush with research was when he was accepted into the Ronald McNair research program at the University of Rochester during his sophomore year. His research project was with Dr. James Ison working on sound localization. After college he joined Dr. Jean Bidlacks laboratory in the Pharmacology department at the University of Rochester Medical School through the PREP program. After a year of research with Dr. Bidlack he was admitted to the University of Texas Health Science Center and joined Dr. Henry Strobel's laboratory to work on cytochrome P450s.

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