MECHANISMS OF INTERFERON MEDIATED DOWNREGULATION OF CYTOCHROME P450 ENZYMES

by

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ABSTRACT

Interferon(IFN) and interferon inducers depress the hepatic and extrahepatic expression of constitutive and inducible cytochrome P450s through a pretranslational mechanism which downregulates the mRNA encoding specific P450 apoproteins. The IFN effect is independent of the mechanism by which the P450 is induced but requires the *de novo* synthesis of an unknown intermediate protein. We investigated the possible pretranslational mechanisms by which IFNα/β (induced with polyIC, 10 mg/kg) depresses P450 expression by examining the degradation of induced CYP1A1 and CYP1A2 mRNAs after inhibiting *de novo* transcription with actinomycin D (1 mg/kg). Steady state mRNAs analyzed by Northern and slot blotting showed that polyIC significantly augmented the loss of CYP1A1 and CYP1A2 mRNA. We used a genetically engineered cell line to show a role for the nucleus in CYP1A1 downregulation by recombinant IFN. We further characterized the pretranslational loss of P450s by performing nuclear run-on transcription assays with nuclei isolated from the liver of male rats. We examined the effect of IFNα/β on the rates of transcription of CYP1A1 and CYP1A2 genes. The results showed that polyIC decreased the rate of transcription of both CYP1A1 and CYP1A2 suggesting that IFNα/β production also depresses gene transcription. The results of these studies suggest that transcriptional as well as posttranscriptional events could account for the pretranslational mechanism by which IFN downregulates P450 expression.

PUBLICATIONS

Parts of the work presented in this thesis have been published in the following peer-reviewed journals.

1. Delaporte, E., Cribb, A.E. and Renton, K.W. (1993). Modulation of rat hepatic CYP3A1 induction by the interferon inducer polyinosinic acid-polycytidylic acid (polyIC). Drug Metab. Dispos. 21:520-523.

2. Cribb, A.E., Delaporte, E., Kim S.G., Novak, R.F. and Renton, K.W. (1994). Regulation of cytochrome P4501A and cytochrome P4502E induction in the rat during the production of interferon α/β. J. Pharmacol. Exp. Ther. 268:487-494.

3. Delaporte, E., Cribb, A.E. and Renton, K.W. Interferon mediated changes in the expression of CYP1A1 in human B lymphoblastoid (AHH-1 TK+/-) cells. Can. J. Physio. Pharmacol. (accepted October 1995).

ABBREVIATIONS AND SYMBOLS

Act D	actinomycin D
AU	arbitrary unit
BNF	β-naphthoflavone
BSA	bovine serum albumin
CYP_	cytochrome P450_
DBA	dibenz(*a,h*)anthracene
DMSO	dimethylsulfoxide
dsRNA	double-stranded RNA
EBV	Epstein Barr virus
EDTA	ethylenediaminetetraacetic acid
EROD	ethoxyresorufin 0-deethylase
G3PD	glyceraldehyde 3-phosphate dehydrogenase
IFN_	interferon_
IL_	interleukin_
kb	kilobase
kDa (kd)	kilodalton
mRNA	messenger ribonucleic acid
PBS	phosphate-buffered saline
PCN	pregnenolone 16α-carbonitrile
PMSF	phenylmethylsulfonyl fluoride
PolyIC (pIC)	polyinosinic acid-polycytidylic acid
PolyT	oligodeoxythymidylic acid
P450	cytochrome P450
RNA	ribonucleic acid
rpm	revolutions per minute

RPMI 1640	RPMI medium 1640 without L-glutamine
SSC	sodium chloride/sodium citrate
SSPE	sodium chloride/sodium phosphate/EDTA
TAO	troleandomycin
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEMED	N,N,N,N'-tetramethyl ethylene diamine
TGF_	transforming growth factor_
TKM	Tris/potassium chloride/magnesium chloride
TNF_	tumor necrosis factor_
U	unit

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INTRODUCTION

1.1 Discovery and Historical Review

In 1958, Klingenberg and Garfinkel, independently reported the occurrence in liver microsomes of a CO-binding pigment with a characteristic optical absorption at 450 nm. A few years later, Omura and Sato (1962) presented evidence on the hemoprotein nature of the pigment. The pigment was identified as a new b-type cytochrome and the name "P-450" (a pigment with an absorption at 450 nm) proposed. Further work by Omura and Sato (1964a, 1964b) led to the characterization of the basic properties of this new hemoprotein. Physiological function of P450 as an oxygen-activating terminal oxidase in various oxygenation reactions was reported by Estabrook *et al* (1963). The role of P450 in the oxidation of drugs by hepatic microsomes was further confirmed by the earlier discovery of enzyme induction (increase in the level of one or more P450s following treatment with certain drugs (Sato and Omura, 1978; Okey, 1990). Using specific enzyme inducers, it became apparent that the rates of metabolism of different compounds did not always increase by the same extent when animals were treated with different inducers. These observations, coupled with the broad substrate specificity of the P450-mediated metabolism, led to speculations about multiple forms of the P450.

Sladek and Mannering (1966) reported the existence of a different P450 species in hepatic microsomes from 3-methylcholanthrene treated rats. By the mid-1970s, the existence of multiple forms of hepatic microsomal P450 was confirmed in protein purification studies (Levin *et al*, 1974; Guengerich, 1977). The term P450 now refers to a superfamily of hemoproteins which are thought to evolve from one ancestral gene many years ago (Nelson *et al*, 1993).

1.1.1 Cytochrome P450

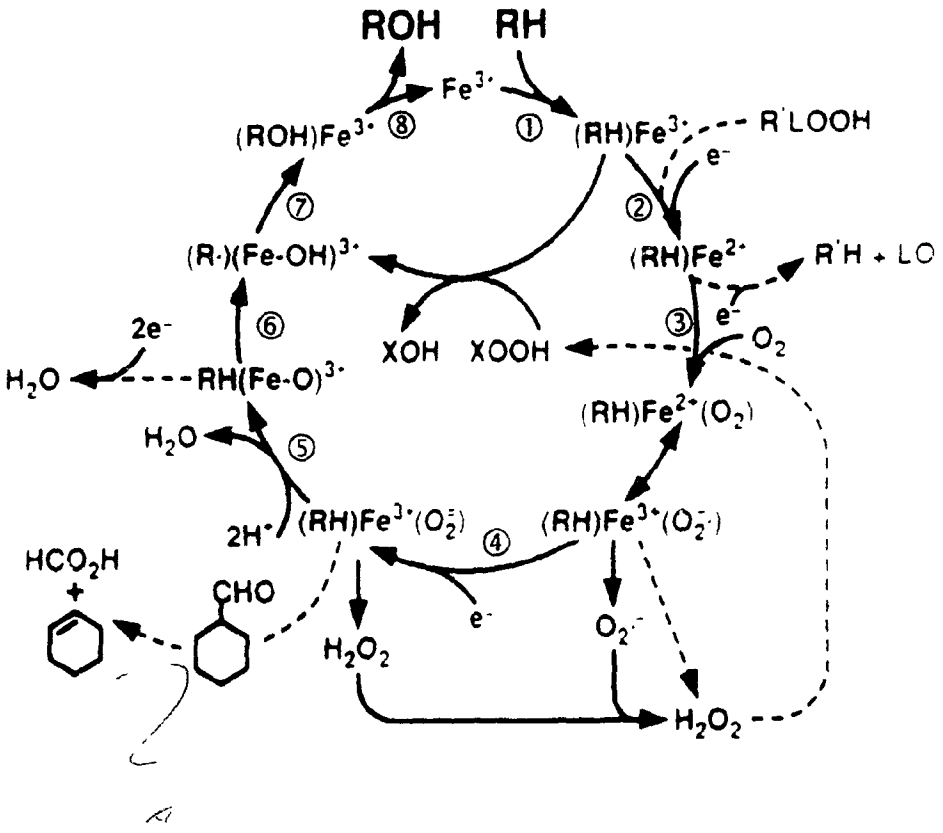
Cytochrome P450s (P450s) are heme-thiolate proteins which catalyze the metabolism of a wide range of endogenous and exogenous substrates. These substrates include physiologically important molecules, such as steroids, eicosanoids, fatty acids, lipid hydroperoxides, retinoids and other lipid metabolites and xenobiotics such as drugs, alcohols, procarcinogens, antioxidants, organic solvents, anesthetics, dyes, pesticides, odorants and flavorants (Gonzalez, 1989; Porter and Coon, 1991; Coon *et al*, 1992). P450s are present in microorganisms, plants and animals. All mammalian tissues examined contain one or more of the enzymes in various organelles, predominantly in the endoplasmic reticulum and mitochondria. Mammalian P450s can be subdivided into two major classes: those involved in biosynthesis of steroids and bile acids, and those that metabolize xenobiotics (Gonzalez,

1992). It is thought that the older steroidogenic P450s evolved in early organisms to synthesize steroids needed for membrane integrity and later in highly differentiated organisms, as hormonal mediators of development (Nebert, 1991). Xenobiotic-metabolizing (mainly microsomal) P450s probably evolved from the steroidogenic P450 to oxidatively degrade hydrophobic dietary components that could not be easily eliminated from animals (Nelson and Strobel, 1987; Nebert *et al*, 1989; Gonzalez and Nebert, 1991). These P450 genes may have evolved to detoxify foreign chemicals that animals ingest daily.

The P450 catalyzed reaction represented schematically in Fig 1 is thought to involve the following steps:

1. binding of substrate and a shift in the spin state of oxidized P450 from the low to the high spin state;
2. reduction of the flavin prosthetic groups of P450 reductase by NADPH.
3. transfer of one of two available electrons to P450;
4. binding of molecular oxygen to give a ferrous-P450-dioxygen complex;
5. transfer of a second electron from P450 reductase to the complex;
6. cleavage of the oxygen molecule with incorporation of one oxygen

Figure 1



Cytochrome P450 catalytic cycle. Schematic representation of the cytochrome P450 catalytic cycle. Fe^{3+} represents heme iron at the active site. RH is the substrate and ROH the monooxegenated product. R'LOOH represents a lipid hydroperoxide and R'H and LO the corresponding reduction products. XOOH represents a peroxy compound that serves as an alternate oxygen donor to molecular oxygen (taken from Coon *et al*, 1992).

atom into a molecule of water;

7. transfer of the second oxygen atom to the substrate and dissociation of the product (Ortiz de Montellano, 1986; Porter and Coon, 1991; Coon *et al*, 1992).

The scheme also shows:

1) the release of products of oxygen reduction that are not coupled to substrate hydroxylation.

2) the peroxide shunt in which a peroxy compound donates the oxygen atom for substrate hydroxylation with no requirement for molecular oxygen or for NADPH as an electron donor.

3) the ability of ferrous P450 to donate electrons in a stepwise manner to promote reactions under anaerobic conditions.

Most P450-mediated biotransformations result from the insertion of one atom of oxygen, derived from O_2, into a substrate (monooxygenation). Monooxygenation frequently produces unstable intermediates which undergo molecular rearrangement to give rise to different reactions, including oxidative and reductive dehalogenation; N-hydroxylation and N-oxidation; oxidative deamination; S-, N-, and O-dealkylation, aliphatic and aromatic hydroxylation (Gonzalez, 1989). The reactions known as Phase I or functionalization reactions serve to convert lipophilic substrates to more polar compounds that

can easily be excreted directly or after conjugation with water-soluble molecules in Phase II reactions. In addition to detoxication reactions, P450s sometimes convert foreign compounds to metabolites with greater cytotoxicity, mutagenicity, or carcinogenicity (Gonzalez, 1989; Porter and Coon, 1991). Microsomal drug oxidations also play a role in converting some drugs to more pharmacologically active metabolites or prodrugs to active drugs (Low and Castagnoli, 1991). Xenobiotic-metabolizing P450s in contrast to steroidogenic P450s display broad and overlapping substrate specificities with poorer structural conservation between species (Porter and Coon, 1991).

1.1.2 Nomenclature

The molecular cloning and subsequent isolation and sequencing of P450 proteins, cDNAs and genes have led to a P450 classification system based on primary amino acid sequence alignment data (Nebert *et al*, 1987). The suggested system of naming based on divergent evolution of the P450 superfamily recommends a P450 gene or cDNA include the italicized root "*CYP*", denoting *cy*tochrome *P*450, an Arabic number designating the P450 family, a letter indicating the subfamily when more than one subfamily exists within the family and an Arabic number representing the individual gene (Nebert *et al*, 1987; Nebert *et al*, 1991; Nelson *et al*, 1993). A P450 protein

sequence from one gene family usually shares ≤40% amino acid identity to a P450 from another family. P450s within the same subfamily always exhibit >55% sequence identity.

1.1.3 The Cytochrome P450 Superfamily

Cytochrome P450s are thought to be very ancient, having evolved from an ancestral gene which existed more than 3.5 billion years ago, a time that predates drugs, animal-plant interactions and combustion of organic matter (Nelson *et al,* 1993). It is likely that the enzymes evolved after superoxide dismutase to participate in oxidative and peroxidative metabolism of endogenous molecules and environmental chemicals utilized for energy. The gene superfamily consists of more than 221 genes and 12 putative pseudogenes. They were described in 31 eukaryotes and 11 prokaryotes. Twelve of the 36 gene families described exist in all mammals examined. Of the P450 families described to date, families 1, 2, 3 and 4 encode for catabolic microsomal enzymes found primarily in the liver and some extrahepatic tissues. CYP 7, 11, 17, 19, 21, 22 and 27 families code for some of the P450s involved in steroidogenic and cholesterol metabolism. The P450s involved in steroid biosynthesis are expressed in specialized extrahepatic tissues. Also, involved in steroid biosynthesis are the mitochondrial P450s. They belong to the CYP11

family. The enzymes are expressed in the adrenal cortex and use adrenodoxin and adrenodoxin reductase for electron transfer (Gonzalez, 1989; Nelson *et al*, 1993).

1.1.4 Regulation of Cytochrome P450s

Many factors, including genetic constitution, diet, alcohol intake, cigarette smoking, age, disease, hormone levels and exposure to xenobiotics, determine the P450 levels in an individual. The fascination with P450 induction by drugs and other xenobiotics played a major role in stimulating interest in the regulation of P450 expression. In keeping with enzyme multiplicity, a number of different mechanisms have been implicated in P450 regulation (Porter and Coon, 1991; Coon *et al*, 1992). Modulation of gene transcription is the most common regulatory mechanism. Posttranscriptional regulation occurs at the level of RNA processing, mRNA stabilization, translation, protein stabilization, or degradation. Transcriptional and posttranscriptional regulation of CYP2E1 gene expression are well known. Transcriptional activation of the gene occurs at birth and in the adult by fasting (Johansson, 1990). The development of diabetic state induces a 10-fold increase in mRNA due mainly to stabilization (Song *et al*, 1987), whereas chemical inducers such as acetone, ethanol and imidazole induce 2E1 protein

stabilization without affecting the mRNA (Eliasson *et al*, 1990).

Many P450s are subject to tissue-specific expression with resultant differences in isozyme composition and activity in various tissues (Porter and Coon, 1991; Okey *et al*, 1994). Developmental and hormonal regulation of P450 expression have also been described (Jansson *et al*, 1985; Waxman *et al*, 1988). In rodents, sex-specific expression of certain P450s by neonatal imprinting and by hormonal regulation in mature animals is dependent, in part, on steroid hormones and pituitary growth hormone (Gonzalez, 1992b). The P450s involved in steroid biosynthesis are regulated, in part, by adrenocorticotrophic hormone which acts through cAMP to increase gene transcription (Simpson and Waterman, 1988; Simpson *et al*, 1990).

To date, nuclear receptors have been implicated in the expression of only two gene families, namely CYP1 and CYP4A. Despite the mechanistic similarities these receptors share with the nuclear hormone and receptors, no endogenous ligands have been described for any of them (Poellinger *et al*, 1992). The aryl hydrocarbon (Ah) or dioxin receptor belongs to a family of basic helix-loop-helix DNA binding proteins. It binds polycyclic aromatic hydrocarbons, polychlorinated biphenyls and other related ligands to activate the transcription of CYP1A1 and to an extent, CYP1A2 and 1B1 (Okey *et al*, 1994). Peroxisome proliferators are ligands for the peroxisome proliferator-

activated receptor (PPAR) which mediates the induction of CYP4A family isozymes. PPAR is a member of the steroid-thyroid-retinoic acid receptor superfamily.

1.1.5 Drug Metabolizing Cytochrome P450s

Cytochrome P450s in gene families 1-4 have been shown to be involved in the metabolism of drugs and other xenobiotics (Gonzalez *et al*, 1993). The enzymes have been characterized in bacteria, yeast, fish, chickens, rats, mice, hamsters, rabbits and humans. In humans, drug metabolism is performed mostly by CYP1A2, CYP2D6, CYP2E1, CYP2C9, CYP2C19 and CYP3A4. Table 1 shows the percentage expression of drug metabolizing P450s based on the amount of apoprotein and activity in the liver (Shimada *et al*, 1994).

1.1.5.1 CYP1 Gene Family

The CYP1A subfamily codes for two proteins (1A1 and 1A2) which are active in the metabolism of polycyclic hydrocarbons, aromatic amines and heterocyclic amines. The two enzymes share 75% amino acid sequence homology and have similar gene organization (Fujii-Kuriyama *et al*, 1992). CYP1A1 and 1A2 genes are ubiquitous and well conserved in mammals. It was suggested that the enzymes may be essential for survival and may play key

Table 1

Percentage expression of hepatic drug-metabolizing P450s.

Cytochrome P450	% Total P450
3A	30
2C	20
1A2	13
2E1	7
2A6	4
2D6	2
2B6	<.5
Others	23

Based on substrate-specific cytochrome P450 activities and immunochemical determination of apoprotein by Shimada *et al* (1994) in the liver microsomes of 30 Japanese and 30 Caucasians.

roles in the metabolism of critical endogenous substances. There is now evidence to support involvement of the AhR in the development of liver and immune system (Kimura, *et al* 1987; Fernandez-Salguero *et al*, 1995). CYP1A1 is an inducible P450 that is expressed in the liver and many extrahepatic tissues, such as lung, skin and kidney. Compared to other P450s, the regulation of this gene is very well characterized. The gene is activated transcriptionally by ligands that exert their effect by binding to the Ah receptor. Ligand binding converts the receptor into a form that translocates to the nucleus and is capable of binding specific DNA sequences located upstream of the CYP1A1 gene. These DNA enhancer sequences are variously referred to as Ah responsive elements (AhREs), dioxin-responsive elements/drug regulatory elements (DREs) or xenobiotic responsive elements (XREs) (Gonzalez *et al*, 1993; Okey *et al*, 1994). Three to five AhREs are found in the mouse, rat and human CYP1A1 genes. These contain the core sequence TCACGC, which is preceded by seven and followed by two base pairs that are required for receptor binding and/or activity. Interaction of the receptor complex with AhRE induces the transcription of at least 26 genes whose products are involved in cell growth and differentiation or drug metabolism.

In addition to CYP1A1, other drug metabolizing enzymes regulated by the AhR include CYP1A2 (in part), CYP1B1, glutathione-S-Transferase Ya, the

TCDD inducible UDP-glucuronosyltransferase UGT1*06, NAD(P)H:quinone oxidoreductase NQO_1 and aldehyde dehydrogenase ALDH3c. CYP1A1 expression is also under the control of a basic transcriptional element (BTE) (Fujii-Kuriyama *et al*, 1992; Imataka *et al*, 1992) and a negative regulatory element (NRE) (Boucher *et al*, 1993; Boucher and Hines, 1995).

The CYP1A2 gene is expressed constitutively (in the absence of an inducer) in the liver and shows marked induction on exposure to AhR ligands. Much less is known about the mechanism that controls the expression of CYP1A2 gene. The AhR has been implicated in both transcriptional and posttranscriptional induction of the enzyme. A recent report described Ah receptor-specific and promoter-specific elements that regulate the expression of human CYP1A2 gene, suggesting the involvement of AhR as well as AhR independent mechanisms in CYP1A2 induction (Quattrochi *et al*, 1994).

CYP1A1 metabolizes benzo(a)pyrene and other substrates, while 1A2 is active towards 2-acetylaminofluorine and other arylamine compounds, aflatoxin B_1 and promutagens derived from pyrolysates of amino acids and proteins (Gonzalez *et al*, 1993). CYP1A2 also metabolizes caffeine, theophylline and phenacetin. The CYP1A subfamily is well known for its ability to convert procarcinogens to mutagenic derivatives and its role in chemical carcinogens (Boucher and Hines, 1995).

A new member of the CYP1 gene family, CYP1B1 has recently been described (Pottenger *et al*, 1991; Sutter *et al*, 1994). cDNA cloning and sequence analysis of the mouse, rat and human CYP1B1 have been published (Savas *et al*, 1994; Sutter *et al*, 1994; Walker *et al*, 1995). The mouse and rat share 92% amino sequence similarity. The amino acid sequence of the human CYP1B1 is 80% and 81% identical to the respective rat and mouse sequences. Walker *et al* (1995) characterized CYP1B1 expression in Sprague-Dawley rats and found that the enzyme is constitutively expressed in the adrenal glands and to a lesser extent, the testes of rats. Following treatment with TCDD, CYP1B1 mRNA was induced in the liver and kidney in a sex-dependent manner, with female rats showing significantly higher levels than similarly treated males. Based on the constitutive expression of CYP1B1 in the adrenals and testes it has been suggested that the enzyme may have endogenous physiological functions. The role of CYP1B1 in drug metabolism and activation of procarcinogens remains to be determined. There are reports of other polycyclic aromatic hydrocarbon inducible P450s whose identities await further characterization (Weaver *et al*, 1994).

1.1.5.2 CYP2 Gene Family

1.1.5.2.1 The CYP2A Subfamily

Three isozymes CYP2A1, 2A2 and 2A3 have been described in the rat. CYP2A1 shares 93% nucleotide sequence and 88% amino acid sequence with CYP2A2. The enzymes exhibit positional specificities towards testosterone hydroxylation. CYP2A3 is expressed in the lung following methylcholanthrene induction. The enzyme shows 71% and 73% amino acid sequence similarity to CYP2A1 and 2A2, respectively. In humans three orthologous P450s; CYP2A6, 2A7 and 2A13 have been described. CYP2A6 constitutes up to 5% of total hepatic P450. The enzyme is involved in the metabolic activation of procarcinogens present in tobacco smoke or food. Coumarin and fadrozole are mainly metabolized by CYP2A6 (Gonzalez, 1992b; Raunio *et al*, 1995).

1.1.5.2.2 The CYP2B Subfamily

Members of the CYP2B family are highly inducible by phenobarbital. In the rat, CYP2B1 and 2B2 induction occur through transcriptional activation (Waxman and Azaroff, 1992). The enzymes are closely related in structure exhibiting 97% amino acid similarity. They show similar but distinguishable preferences for specific substrates. CYP2B2 protein is expressed at low but detectable levels in the uninduced rat liver. 2B1 protein expression is at least

5-10 fold lower and undetectable in the hands of many workers. The enzymes metabolize a broad range of lipophilic drugs and steroidal substrates, including androgens (Waxman and Azaroff, 1992). A third rat 2B subfamily member designated 2B3 exists. It is constitutively expressed in male and female liver and is not inducible with phenobarbital. CYP2B3 shares 77% amino acid sequence homology with 2B1 and 2B2 (Gonzalez, 1992b).

In humans, two enzymes have been described. CYP2B6 is expressed in the liver and metabolizes cyclophosphamide (Yamano *et al*, 1989). CYP2B7 is a seleno-containing protein which is mainly expressed in the lung (Yamano *et al*, 1989; Czerwinski *et al,* 1994).

1.1.5.2.3 The CYP2C Subfamily

A total of at least nine CYP2C subfamily isozymes have been cloned in the rat. In humans, five distinct genes - 2C8, 2C9, 2C17, 2C18 and 2C19 - have been identified. The genes encode P450s which metabolize several clinically important drugs. CYP2C8 and 2C9 metabolize tolbutamide and mephenytoin. CYP2C9 also metabolizes warfarin and phenytoin. CYP2C19 is the principal enzyme responsible for mephenytoin metabolism in humans. Other 2C19 substrates may include omeprazole, propranolol, proguanil, imipramine, diazepam and some barbiturates (Cholerton *et al*, 1992;

Gonzalez, 1992b; Demorais *et al*, 1994). The CYP2C subfamily genes share 80-95% homology at the nucleotide level. Genetic polymorphisms have been characterized for mephenytoin metabolism. The principal defect occurs in 2C19 expression. There is also evidence for defects in CYP2C9 expression in certain individuals (Veronese *et al*, 1993). Most members of the CYP2C subfamily are expressed constitutively. A few, however, are induced by phenobarbital. In addition, the enzymes are under developmental and sex-specific regulation (Gonzalez, 1992b).

1.1.5.2.4 The CYP2D Subfamily

Five CYP2D genes exist in the rat. CYP2D1, 2D2, 2D3, 2D4 and 2D5 are constitutively expressed in the liver and kidney. The human CYP2D cluster consists of the three genes, namely CYP2D6, CYP2D7P and CYP2D8P. Only CYP2D6 gene produces detectable mRNA transcripts in the liver (Cholerton *et al* 1992; Gonzalez, 1992b). The CYP2D locus is highly polymorphic, yielding at least 17 different haplotypes. Debrisoquine and sparteine are commonly used as probe drugs to assess CYP2D6 activity. Using debrisoquine, three phenotypes can be discerned in the population: extensive metabolizers, poor metabolizers and ultrarapid metabolizers (Bertilsson *et al*, 1995). The molecular basis for this variation in metabolism is inherent in the

distribution of several different functional and nonfunctional 2D6 alleles.

CYP2D6 metabolizes a wide range of therapeutic classes of drugs, including several antiarrhythmic drugs, β-adrenoceptor blockers, antihypertensives, neuroleptics, tricyclic antidepressants, MAO inhibitors, morphine derivatives, codeine and dextromethorphan, phenformin, perhexilene and methoxyamphetamine.

1.1.5.5 The CYP2E Subfamily

A single CYP2E gene exists in humans, rats and mice. Rabbits have two very similar genes - CYP2E1 and 2E2. The enzyme is well conserved and displays similar catalytic activities across species, suggesting an important physiological role. A physiological role for CYP2E1 has been defined in its involvement in gluconeogenesis during fasting (Casazza *et al*, 1984).

CYP2E1 is a major xenobiotic-metabolizing enzyme that is capable of activating numerous low molecular-weight toxins and carcinogens. It is involved in the metabolism of substrates such as ethanol, acetone, acetol, acetoacetate, diethyl ether, p-nitrophenol, halothane, enflurane, sevoflurane, isoflurane, methoxyflurane, acetaminophen, benzene, pyridine and N-nitrosodimethylamine (Gonzalez, 1992b; Kharasch and Thummel, 1993).

1.1.5.3 CYP3 Gene Family

The CYP3A family of P450s are found in rats, rabbits, mice, gerbils and humans (Babany *et al*, 1988). The enzymes are inducible with glucocorticoids, antiglucorcorticoids, phenobarbital, imidazole antifungal agents and macrolide antibiotics. The imidazoles (Yee and McGuire, 1990; Halpert *et al*, 1994) and the macrolides (Babany *et al*, 1988) are also inhibitors of the enzymes. In rats, CYP3A expression is sex-dependent, with high constitutive levels of CYP3A2 present in adult male rats. In female rats, CYP3A1 is present at birth, but drops to very low levels by the time of sexual maturation. In the adult female rat, the enzyme is absent, but inducible with prototype inducers. In humans, CYP3A subfamily P450s, namely, CYP3A3, CYP3A4, CYP3A5 and CYP3A7 have been described. CYP3A3, 3A4 and 3A5 are expressed in varying amounts in the liver with CYP3A4 being the most abundant form (Cholerton *et al*, 1992; Gonzalez, 1992a). CYP3A3 has the same catalytic activity as CYP3A4 and has only eleven amino acid substitutions. CYP3A5 has similar substrate specificity as CYP3A4 and is expressed in only 10-20% of adult human livers examined. CYP3A7 is expressed in only human fetal liver. In addition to hepatic expression, the CYP3A enzymes are also found in human extrahepatic tissues, including the gastrointestinal tract (McKinnon *et al*, 1995). Regulation of the liver enzymes is controlled by transcriptional and posttranscriptional

mechanisms (Gonzalez, 1992b). With regards to drug metabolism, CYP3A4 is the single most important P450. It metabolizes more than 55 different clinically used drugs which include cyclosporin, erythromycin, verapamil, nifedipine and other dihydropyridines, midazolam, triazolam, lidocaine, the contraceptive agent 17α-ethynylestradiol and quinidine. Other 3A4/5 substrates include aldrin, caffeine, benzphetamine and steroids such as cortisol, testosterone and 17β-estradiol.

Members of the CYP3A subfamily play a key role in the bioactivation of carcinogens, including benzo(a)pyrene and aflatoxin B_1 and the conversion of hexachlorobenzene to a porphyrinogenic derivative. It has recently been shown that the enzymes are capable of converting a heterocyclic amine, 2-amino-3,4-di-methylimidazo[4,5-f] quinoline to a mutagen in the presence of dietary flavonoids (Lown *et al*, 1992; McKinnon *et al*, 1995).

1.1.5.4 CYP4 Gene Family

Hypolipidemic drugs clofibrate, ciprofibrate, nafenopin and other peroxisome proliferators activate the transcription of the CYP4 family. The enzymes perform microsomal ω-hydroxylation of fatty acids. Together with peroxisomal β-oxidations, the net result is an increase in mitochondrial-

independent β-oxidation of fatty acids (Poellinger *et al*, 1992). At least 17 CYP4 proteins have been described in mammals including rats and humans. The CYP4A genes are expressed primarily in the liver and kidney (Gonzalez *et al*, 1989). Apart from the physiological role in fatty acid metabolism, no exogenous substrates have been described for the human enzymes. In the rat, however, CYP4B1 has been shown to metabolically activate 2-aminofluorene and 4-ipomeanol (Gonzalez, 1992b).

1.2 Interferon and Hepatic Drug Metabolism

1.2.1 Interferons

Interferons (IFNs) belong to a heterogenous family of glycoproteins whose production is stimulated by viral infection, intracellular parasites, bacteria, bacteria endotoxins, synthetic RNA polymers and some low molecular weight chemicals (Singh *et al*, 1982). They were discovered in 1957 by virtue of their ability to disrupt viral replication (Sen and Lengyel, 1992; Landolfo *et al*, 1995). IFNs are now known to induce a large number of genes whose products either singly or corporately mediate their antimicrobial, antigrowth and immunomodulatory effects (Constantoulakis *et al*, 1993). On the basis of their genomic organization and protein structure, type of inducer and cell sources, the IFNs are classified as IFNα, IFNω, IFNβ and IFNγ. IFNα and

IFNω (leukocyte IFNs), together with IFNβ (fibroblast IFN), are designated as Type I IFN superfamily because their genes are normally clustered without intervening sequences on a single chromosome. IFNγ (immune IFN) is designated as Type II IFN. Ovine trophoblast protein I and bovine trophoblast protein I have recently been included in the IFN system as IFNτ (trophoblast IFN) (Sen and Lengyel, 1992; Landolfo *et al*, 1995).

1.2.2 The Interferon Receptors and Signal Transduction

IFNs exert their biological actions by interacting with specific cell surface receptors. IFNα and IFNβ bind to the same receptor designated Type I or IFNα/β receptor. IFNγ acts through the Type II IFN receptor. IFNs bind to their receptors in a species-specific manner and activate the transcription of several genes via multiple signal transduction pathways. Two contrasting models have been proposed for IFN signal transduction. That is IFN signalling involves activation of second messenger systems that stimulate transcription of IFN-inducible genes and the direct activation of a cytoplasmic transcription complex without the need for second messengers (Landolfo *et al*, 1995). The molecular events leading to gene transcription by IFNα include receptor-specific ligand activation of latent cytoplasmic proteins by phosphorylation, translocation of these proteins to the nucleus to join the multiprotein complex,

interferon-stimulated gene factor 3 (ISGF3) and binding of ISGF3 to the interferon-stimulated response element (ISRE) (Shuai *et al*, 1992). ISRE is a consensus cis-acting DNA sequence which confers inducibility by IFNα/β and is present in the 5'-flanking region of all IFNα/β-inducible genes (Sen and Lengyel, 1992). IFNγ-mediated gene expression involves the transcription of a gene encoding a guanylate binding protein (GBP) by activating a latent cytoplasmic factor, GAF. GAF translocates to the nucleus, binds to the gamma-activated site (Shuai *et al*, 1992) and directs the transcription of IFNγ-inducible proteins.

1.2.3 Interferon Inducible Proteins

IFNs induce at least 30 different proteins. Some of these are induced by all types of IFNs, whilst others are preferentially or exclusively induced by IFNα/β or IFNγ (Sen and Lengyel, 1992). Induction may result in the appearance of previously undetectable proteins in cells, or slight increases in the level of preexisting proteins. Several of these proteins are enzymes, others are transcription factors, immune mediators (major histocompatibility complex class I and II and β_2-microglobulin) antiviral Mx proteins. The biological effects of IFNs are mediated by one or more of these proteins. For example, the antiviral effect is attributed in part to the induction of P1 kinase, 2',5-

oligoadenylate synthetase, indoleamine 2,3-dioxygenase, Mx proteins, the 9-27 protein and nitric oxide synthase (Kurapiah *et al*, 1993).

1.2.4 Interferon Inducers

IFNs are induced by a wide variety of agents, including virus, bacteria, chlamydia, rickettsiae, protozoa, mycoplasma, bacteria endotoxin, bacteria and fungal cellular extracts, synthetic RNA polymers, non-RNA polymers, including polycarboxylates and polyposphates, low molecular weight compounds such as tilorone and anthraquinones, cytokines, mitogens and antigens (including tetanus, diphtheria toxoids, bacteria, viruses). Endotoxins and other bacterial and fungal extracts induce IFNα, IFNβ and IFNγ. IFNγ is produced solely by mitogens and antigens. All the other inducers stimulate the production of IFNα and IFNβ (Torrence and De Clercq, 1977; Mannering and Deloria, 1986; De Maeyer and De Maeyer-Guignard, 1988).

1.2.5 Modulation of P450 Drug Metabolism by Interferon

In 1972, Moraiian *et al* reported depression of hepatic drug metabolism as a side effect accompanying the antiviral action of polyIC. Other IFN inducers including tilorone, quinacrine and viruses were later shown to depress hepatic P450 and related biotransformation reactions (Renton and Mannering,

1976a; Renton and Mannering, 1976b). Studies with recombinant IFNs have confirmed the role of IFN in P450 depression following treatment of experimental animals with IFN-inducers (Parkinson *et al*, 1982; Singh *et al*, 1982; Taylor *et al*, 1983; Franklin and Finkle, 1985; Moochhala *et al*, 1989). Chang *et al* (1978) documented alteration of theophylline clearance in humans during acute respiratory viral illness. This impairment of theophylline elimination was thought to result from IFN production by the virus (Renton, 1978). There are many examples of altered drug metabolism in infected humans and several reports of altered drug clearance during clinical treatment with recombinant IFNs (Renton, 1993). The first evidence that IFN inhibits hepatic drug metabolism was provided by Williams and Farrell (1986). They showed that a single injection of IFN-α2a depressed antipyrine clearance in patients with chronic active hepatitis B. In another study in hepatitis patients and healthy volunteers Williams *et al* (1987) demonstrated a reduction in the elimination of theophylline 24 hours after IFN administration. Okuna *et al* (1990) reported the direct depression of hepatic drug metabolizing enzyme activity in humans treated with IFNα.

The development of specific substrate assays to measure P450 activity, polyclonal antibodies for P450 apoprotein detection and knowledge of P450 cDNA sequences have allowed examination of the effects of IFN on the

expression of specific P450 isozymes. The expression of constitutive enzymes, such as CYP3A2 (Craig *et al*, 1990), CYP2C11 (Morgan and Norman, 1990), CYP2C12 (Morgan, 1991), CYP2E1 (Sakai *et al*, 1992), in the rat and CYP1A2 and CYP2C6 (Stanley *et al*, 1991) in the mouse are shown to be downregulated by IFNs and/or their inducers. On the contrary, IFNs did not appear to affect the expression of inducible CYP3A1 in the female rat (Craig *et al*, 1990) and the expression of inducible CYP1A1, CYP1A2, CYP26C and CYP2B1 in the mouse (Stanley *et al*, 1991). These findings led to the suggestion that induction of P450 may provide protection against IFN-mediated downregulation of P450s. In studies where endogenous IFNα/β production was stimulated by polyIC the downregulation of inducible hepatic P450s including CYP4A1 (Renton & Knickle, 1990), CYP1A1, CYP1A2 and CYP2E1 (Cribb *et al*, 1994) in the rat, CYP2B isozymes in the mouse and rat (Anari *et al*, 1995) and CYP3A1 female rats (described in chapter 3) was demonstrated. Other workers have shown downregulation of P450s by recombinant IFNs in cultured rat (Clark *et al*, 1995) and human and rat (Abdel-Razzak *et al*, 1994; Abdel-Razzak *et al*, 1995) hepatocytes.

1.2.6 Modulation of Drug Metabolism by Other Cytokines

Acute phase reaction is a physiological response to tissue damage that is

characterized by local inflammation, fever, alterations in endocrine function, decreased plasma iron and zinc and dramatic changes in proteins produced by the liver, including plasma proteins. The response is known to decrease the levels of various P450s (Stanley *et al*, 1988; Renton and Knickle, 1990). Cytokines, especially interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGFβ) and IFNs mediate the systemic and liver-associated responses (Barker *et al*, 1992). Studies have been carried out to characterize the effect of acute phase reactants on hepatic drug biotransformations. In cell culture experiments, IL-1α, IL-1β, IL-6, TNFα and TGFβ have been shown to suppress CYP1A1 expression (Fakuda et al, 1990; Barker *et al*, 1992; Fakuda and Sassa, 1994; Abdel-Razzak *et al*, 1994; Clark *et al*, 1995;). CYP1A2 expression was shown to be downregulated by IL-1β and IL-6 (Barker *et al*, 1992; Fakuda *et al*, 1990). The enzymatic activity and apoprotein levels of CYP2B1/2 in rat hepatocytes were depressed by IL-1α, IL-1β and IL-6 treatment (Clark *et al*, 1995). Fakuda *et al* (1990) also showed decreased expression by CYP3A3 mRNA by IL-6 in human hepatoma cells. In addition, induction of inflammation by endotoxin or turpentine has been reported to depress the hepatic mRNAs for CYP2C11 and CYP2C12 (Wright and Morgan, 1990).

1.2.7 Mechanisms of Interferon Mediated P450 Downregulation

Although the exact mechanism is not known, there is evidence that the loss of P450 occurs at a pretranslational step leading to the depression of mRNA and a subsequent decrease of apoprotein synthesis (Craig *et al*, 1990; Morgan and Norman, 1990; Renton and Knickle, 1990; Morgan, 1991; Cribb *et al*, 1994). By using protein synthesis inhibitors, evidence was provided for the requirement of *de novo* synthesis of an unknown intermediate protein (Moochhala and Renton, 1991). Recently, it was shown that this pretranslational mechanism is independent of the induction processes (Cribb *et al*, 1994; Anari *et al*, 1995; described in chapter 3). Two mechanisms have been proposed to explain the IFN-mediated depression of cytochrome P450s. Ghezzi *et al* (1984, 1985) proposed a mechanism based on the induction of xanthine oxidase by IFN and IFN inducers. They suggested that increases in hepatic xanthine oxidase activity results in the generation of free oxygen radicals which destroy P450s. Recent evidence, however, has failed to support a role for oxygen radicals in P450 protein degradation, since a decrease in P450 mRNA always precedes the decrease in P450 apoprotein (Craig *et al*, 1990; Morgan and Norman, 1990; Renton and Knickle, 1990; Morgan, 1991; Cribb *et al*, 1994). Mannering *et al* (1988) and more recently, Cribb and Renton (1993) have demonstrated that xanthine oxidase is not involved in

P450 downregulation by IFN in the mouse and rat, respectively. It is, however, possible that xanthine oxidase could play a subsidiary role in augmenting P450 loss by increasing P450 degradation.

The second mechanism is based on the induction of antiviral proteins by IFN. Mannering *et al* (1988) suggested initially that IFN-inducible protein kinase and/or 2-5 oligoadenylate synthetase (2'-5'A synthetase) may inhibit the synthesis of P450. Both enzymes are activated by dsRNA. The protein kinase phosphorylates protein initiation factor eIF2 and inhibits peptide chain initiation (Renton and Knickle, 1990; Sen and Lengyel, 1992). Since the description of a pretranslational depression of P450s by IFN, the involvement of protein kinase is no longer tenable. When activated, 2-5A synthetase produces a series of (2'-5')-linked oligoadenylates that activate a latent endonuclease, RNase L, that cleaves single-stranded mRNA after UA, UG, UC and UU residues at the 3' end (Floyd-Smith *et al*, 1981; Salehzada *et al*, 1993). RNase L could degrade mRNAs coding for P450 since its cleavage sites are likely to be found on mammalian mRNA transcripts. In addition to increased mRNA degradation IFN could also act pretranslationally at the level of gene transcription, RNA processing and RNA transport to downregulate P450 gene expression.

1.2.8 Principal Objectives of the Thesis

The experiments described in this thesis were performed to determine the following:

1. Whether or not the mechanism of P450 induction affects IFN-mediated downregulation of P450.
2. To determine the mechanism or mechanisms of pretranslational downregulation of inducible P450s by IFN and IFN inducers.

2.0 MATERIALS AND METHODS

2.1 Materials

The materials used in the experiments reported in this thesis were supplied by the following companies (common laboratory reagents not included).

Sigma Chemical Company

acetylacetone

acrylamide

alkaline phosphatase conjugated antiboby

bovine serum albumin

bromophenol blue

5-bromo-4-chloro-3-indolyl phosphate (BCIP)

cesium chloride

Coomassie brilliant blue G

Denhardt's solution

dextran sulfate

deoxyribonuclease I (DNase I)

dibenz(*a,h*)anthracene

dimethylsulfoxide

dithiothreitol

erythromycin

ethidium bromide

ethoxyresorufin

ethylenediaminetetraacetic acid (EDTA)

formamide

glucose

glycine

guanidine hydrochloride

guanidine isothiocyanate

iodoacetate

methylene blue

2-mercaptoethanol

nitro blue tetrazolium

nitrocellulose membrane (Dura-Blot™)

N-lauryl sarcosine (sarcosyl)

N,N'-methylene-bis-acrylamide

N,N,N,N'-tetramethyl ethylene diamine (TEMED)

β-naphthoflavone

β-nicotinamide adenine dinucleotide phosphate (NADPH)

phenol

phenylmethylsulfonyl fluoride (PMSF)

polyvinyl-pyrrolidone

polyIC

potassium ferrocyanide

pregnenolone 16α-carbonitrile

resorufin

ribonucleoside triphosphate solutions

ribonucleoside vanadyl complexes

salmon sperm DNA

semicarbazide hydrochloride

sodium dithionate

sodium dodecyl sulphate

sodium potassium tartrate

spermidine

sucrose

troleandomycin

Tween 20

Tween 80

xylene cyanol

GIBCO-BRL

buffer-saturated phenol

DNA ladder (1 kb)

guanidine isothiocyanate

L-glutamine

LB Broth base

T4 polynucleotide kinase

random primers DNA labelling kit

restriction enzymes (*Pst* I, *Hin*d III)

RNA ladder (0.24-9.5 kb)

sodium dodecyl sulfate

Bio-Rad Laboratories

DNA grade agarose

BIO-DOT slot blot apparatus

Mini Sub™ DNA cell

prestained molecular weight standards

titertube®

Boehringer Mannheim

actinomycin D

chloramphenicol

lysozyme (muramidase)

yeast RNA

American Type Culture Collection

human β-actin cDNA

human glyceraldehyde 3-phosphate dehydrogenase cDNA

mouse CYP1A1 cDNA

pUC 18 vector

Gentest Corporation

cHoI and h1A1 v2 cells

horse serum

RPMI 1640 medium

Amersham Canada Ltd.

cytidine deoxynucleoside triphosphate ([α-^{32}P]dCTP)

guanosine ribonucleoside triphosphate ([α-^{32}P]GTP)

Beckman Instruments Inc.

Quick-Seal centrifuge tubes

Bio 101 Inc.

The Geneclean® Kit

DuPont Canada Inc.

adenosine 5'-triphosphate ([γ-^{32}P]ATP)

Fisher Scientific

Dual Mini-vertical Electrophoresis System

Flow Laboratories Inc.

trypan blue

Hoffman-La Roche Ltd

interferonα-2a

Human Biologics Inc.

CYP1A1 antibody

ICN Pharmaceuticals Canada Ltd.

phenol:chloroform saturated solution (25:24:1)

Merck Chemical Company

Coomassie brilliant blue R250

Nalge Company

Nalgene filters (0.2, 0.45 and 0.8μm)

Novopharm Ltd.

ampicillin

OXYgene Dallas

CYP3A1 antibody

Pharmacia Inc.

T4 polynucleotide kinase

2.2 Whole Animal Experiments

2.2.1 Animals and Treatment

Male and female Sprague-Dawley rats (175-225 g), obtained from Charles River Laboratories (Montreal, Quebec) were used. The animals were housed in pairs in plastic cages with clay chips. They were maintained on a 12-hour light/dark cycle under conditions of constant ambient temperature and humidity at the Animal Care Center, Faculty of Medicine, Dalhousie University and allowed free access to Purina rat chow and water *ad libitum*. The rats were kept in our facilities for at least 5 days before use. All animal studies were approved by the Dalhousie University Animal Ethics Committee.

2.2.2 Induction of Cytochrome P450s

Induction of CYP1A enzymes in male rats was accomplished by a single intraperitoneal injection of the prototype inducer β-naphthoflavone (60 mg/kg in olive oil). Induction of CYP3A1 in female rats was by a single intraperitoneal injection of pregnenolone 16α-carbonitrile (300 mg/kg in saline with 0.001% Tween 80) or troleandomycin (200 mg/kg in saline with 0.001% Tween 80). In both cases a 24-hour induction protocol was used.

2.2.3 Induction of IFN

IFNα/β was induced by injecting polyIC (10 mg/kg in saline) intraperitoneally. In experiments where the effect of IFN on induced P450s was investigated, the P450 inducer and polyIC were administered concurrently.

2.3 Preparation of Microsomes

Microsomes were prepared from rat liver by the differential centrifugation method of El Defrawry El Masry *et al*, (1974). After the animals were killed, the livers were harvested, weighed and washed in cold 1.15% KCl. The liver was homogenized in approximately 5 ml of cold KCl/g of liver, with 6-7 strokes of the loose plunger and 3-4 strokes of the tight plunger. The homogenate was centrifuged at 9,000 rpm for 10 minutes at 4°C in a Beckman J2-21 centrifuge. The supernatant was transferred into polycarbonate ultracentrifuge tubes and centrifuged in a Beckman L-60 ultracentrifuge at 40,000 rpm at 4°C for 40 minutes. The supernatant was discarded and the pellet resuspended in an equal volume of glycerol-phosphate buffer (50 mM KH_2PO_4, pH 7.5 containing 20% glycerol and 0.4% KCl) by homogenizing with 7 strokes of the tight plunger. The microsomes were stored in eppendorf tubes at -70°C.

2.4 Microsomal Measurements

2.4.1 Total Microsomal Protein

Total microsomal protein was measured by the method of Lowry *et al*, (1951) using bovine serum albumin as standard.

2.4.2 Cytochrome P450

Total cytochrome P450 in microsomes was determined by the method of Omura and Sato (1964), using a Beckman DU-70 spectrophotometer.

2.4.3 Erythromycin N-demethylase Activity

Erythromycin N-demethylation was estimated as described by Arlotto *et al* (1987). A 1 ml incubation mixture containing potassium phosphate buffer (100 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), semicarbazide (5 mM), NADPH (1 mM), liver microsomes (1.0 nmol P450/ml) and erythromycin (400 μM) was maintained at 37°C. The reaction was initiated by the addition of NADPH and stopped after 30 minutes by the addition of 0.4 ml perchloric acid (17%). The formation of formaldehyde was measured at 412 nm by the method of Nash (1953). Microsomes from TAO-treated rats were decomplexed by adding $K_3Fe(CN)_6$ (20 μM) to the reaction mixture (Babany *et al*, 1988).

2.4.4 Protein Gel Electrophoresis and Western Immunoblot Analysis

Hepatic microsomes were diluted to 20 $\mu g/5\mu l$ with an equal volume Laemmli buffer (162 mM Tris·HCl, pH 6.8, containing 20% glycerol, 0.02% bromophenol blue and 2% SDS). The samples were boiled for 3 minutes and subjected to SDS-PAGE under nonreducing conditions in a vertical slab gel apparatus (Smith, 1984). The separating gel consisted of 7.5% acrylamide in 375 mM Tris·HCL, pH 8.8, containing 0.05% ammonium persulfate and 0.1% TEMED. The stacking gel was 3% acrylamide in 125 mM Tris·HCl, pH 6.8, containing 0.1% SDS, 0.1% ammonium persulfate and 0.2% TEMED. The electrode buffer contained 25 mM Tris·HCl, pH 8.3, 192 mM glycine and 0.1% SDS. Proteins were electrophoretically stacked at 75 V for 15-20 minutes and separated at 125 V for 1-2 hours. Relative molecular weight of proteins were determined by running a prestained SDS-PAGE molecular weight markers (Bio-Rad Laboratories, Hercules, CA) on the same gel.

Following resolution of proteins by SDS-PAGE, they were transferred to nitrocellulose membranes (Towbin *et al*, 1979; Burnette, 1981) by a semi-dry transfer process using the Tyler Research Instrument electrophoretic transfer systems. The transfer buffer contained 25 mM Tris·HCl, pH 8.3, 150 mM glycine, 20% methanol and 0.05% SDS. The gel was allowed to soak in transfer buffer for 30 minutes. At the end of the transfer, the membrane was

incubated with rocking for 1 hour at room temperature in BLOTTO buffer (PBS, pH 7.4, containing 5% $^{w}/_{v}$ low fat Carnation milk and 2% BSA) to block nonspecific binding sites. The membrane was then incubated overnight with rocking at 4°C in an appropriate dilution (1:100-1:1000) of the primary antibody in antibody dilution buffer (BLOTTO buffer containing 0.05% Tween 20). The following day, the membrane was rinsed 3 times for 10 minutes in washing solution (PBS, pH 7.4, containing 0.05% Tween 20), followed by incubation with alkaline phosphatase labelled secondary antibody (1:5000) in antibody dilution buffer. Incubation was for 1 hour at room temperature. The membrane was rinsed and equilibrated in alkaline phophatase assay buffer (100 mM Tris·HCl, pH 9.0, 100 mM NaCl and 50 mM MgCl) for a few minutes. Alkaline phosphatase activity was detected by incubating membrane in alkaline phosphatase assay buffer containing 5-bromo-4-chloro-3-indolyl phosphate (0.017% $^{v}/_{v}$) and nitro blue tetrazolium (0.033% $^{v}/_{v}$) as described by Walker and Gastra (1984). The reaction was terminated after adequate colour development by washing with 20 mM Tris·HCl, pH 8.0, containing 5 mM EDTA. The membrane was then rinsed with distilled water and allowed to air dry. The intensity of the stained bands was determined by densitometry using an Apple OneScanner.

2.5 Isolation and Purification of Total RNA

About 1-2 g of liver was flash frozen with liquid nitrogen, broken into small pieces and stored in cryovials at -70°C. Total RNA extraction and purification was carried out with the necessary precautions to avoid contamination with ribonuclease. The single step guanidine thiocyanate-phenol-chloroform method, described by Chomczynski and Sacchi (1987) was used. RNA concentration was determined spectrophotometrically from the absorbance at 260 nm with a Beckman DU-70. The purity was estimated from the ratio between absorbance at 260 nm and 280 nm (Davis *et al*, 1986). The samples were stored at -20°C.

2.5.1 Agarose Gel Electrophoresis and Northern Blotting

Total RNA (30 μg) was precipitated by adding 3 M sodium acetate and 2.5 volumes of chilled absolute alcohol, denatured by boiling for 2 minutes in 8 mls of gel loading buffer containing 1.6X MOPS buffer (10X MOPS is 0.2 M morpholinopropanesulfonic acid, pH 7.0, 0.05 M sodium acetate, 0.01 M EDTA), 9.6% formaldehyde, 72% formamide, 8% glycerol and 8%$^{v}/_{v}$ of saturated bromophenol blue solution and resolved on a 1% agarose/0.66 M formaldehyde gel using a Bio-Rad Mini-Sub DNA cell as described by Davis *et al* (1986). A 0.24-9.5 kb RNA ladder (Gibco BRL, Life Technologies Inc.,

Burlington, Ontario, Canada) was electrophoresed with the samples to serve as a molecular size marker.

RNA was transferred from the gel to nitrocellulose membrane by the capillary elution method (Davis *et al*, 1986). The gel was rinsed in two changes of 10X SSC (1.5 M NaCl, 150 mM sodium citrate) for 20 minutes each to remove formaldehyde and placed inverted on a piece of Whatman 3MM paper positioned on a solid support with both ends hanging into a reservoir of 10X SSC. The nitrocellulose filter was soaked in water and placed in contact with the gel. This was then covered with two pieces of wet Whatman 3MM paper, six paper towels, four stacks of folded paper towels each 2-3 cm thick and a small weight. RNA was transferred overnight by the ascending flow of 10X SSC. After transfer, the membrane was allowed to air dry and then baked at 80°C for 2 hours to immobilize the RNA.

2.5.2 Preparation of Slot Blots

Total RNA (30 μg/50 μl of sterile water) was denatured by incubating at 60°C for 15 minutes with 50% formamide, 7% formaldehyde and 1X SSC. The samples were cooled on ice and diluted to 10 μg with 400 μl of 20X SSC (3.0 M NaCl, 300 mM sodium citrate). Total RNA was applied onto nitrocellulose membrane using a slot-blot apparatus (Bio-Rad Bio-Dot JM SF

Apparatus, Biorad, Richmond, CA) based on the method of Sambrook *et al*, (1989). The RNA was loaded in columns in 2-fold serial dilutions in 20X SSC starting with 10 μg. The membrane was air dried and baked at 80°C for 2 hours.

2.5.3 Hybridization of Northern and Slot Blots

Prehybridization, hybridization and washing were performed in 50 ml plastic tubes in a hybridization oven. The conditions were chosen to represent the optimal required for the particular radiolabelled probe being used in Northern and slot blot hybridizations. Autoradiography was performed at -70°C for 1-2 days and the band intensities quantitated by densitometry.

2.6 Isolation of Liver Nuclei

The procedure for isolating rat liver nuclei was essentially that of Tukey and Okino (1991) with minor modifications. About 8 g of liver was rinsed in ice-cold homogenization buffer A (50 mM Tris·HCl, pH 7.4, containing 20 mM KCl, 5 mM $MgCl_2$ [TKM] and 500 mM sucrose) and then homogenized in 2 volumes of buffer A using a Potter-Elvehjem glass-Teflon homogenizer (clearance 0.15 mM) with 10 up and down strokes at 1,200 rpm. The homogenate was filtered through four layers of gauze into a 75 ml

polycarbonate centrifuge tube. About 10 ml of ice-cold homogenization buffer B (TKM containing 0.88 M sucrose) was added under the homogenate to form a cushion followed by centrifugation at 4,500 rpm for 15 minutes at 4°C. The supernatant was removed and the crude nuclear pellet vortexed in 0.5 ml of buffer B and then brought to 25 ml with buffer C (TKM containing 2.1 M sucrose). The solution was transferred to a 30 ml Corex tube and centrifuged at 9,500 rpm for 30 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 7 ml of buffer D (TKM containing 2.0 M sucrose and 20% $^{w}/_{v}$ glycerol). This was then layered gently over 3 ml of buffer D in a 12 ml polycarbonate ultracentrifuge tube, followed by centrifugation at 16,200 rpm for 60 minutes at 4°C. The supernatant was removed and the nuclei carefully resuspended in 4 ml of nuclei storage buffer (50 mM Tris·HCl, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 3 mM spermidine and 40% $^{v}/_{v}$ glycerol). An aliquot was diluted with 0.02% toluidine blue and counted in a hemocytometer. The nuclei suspension was stored as 0.3-0.5 ml samples at -70°C.

2.6.1 Binding of DNA Probes to Nitrocellulose Membrane

cDNA plasmid (1 μg) was suspended in enough sterile water to give 90 μl of solution/μg of DNA. The probe was denatured by boiling with 10 μl of 3

M NaOH for 10 minutes and cooling on ice for 5 minutes. For each μg of DNA, 100 μl of 2 M ammonium acetate was added, the solution was vortexed and 200 μl applied to each slot on the nitrocellulose membrane which was previously soaked in 20X SSC for 30-60 minutes using a slot blot apparatus. The location of slots containing DNA was marked with a pencil and the membrane air-dried. The membrane was baked at 80°C for 2 hours, cut into strips that contain the cDNA plasmids and stored in cell culture dishes at room temperature.

2.6.2 Nuclear Run-On Transcription Assay

Frozen nuclei (1-2x10^7/300 μl) were thawed on ice and incubated with 300 μl of reaction mixture containing 20 mM Tris·HCl, pH 7.9, 30% glycerol, 2.5 mM dithiothreitol, 5.0 mM $MgCl_2$, 3 mM spermidine, 4.2 mM each of ATP, CTP and UTP, 130 mM KCl and 250 μCi of [α^{32}P]GTP (Amersham Canada, 3000 Ci/mmol) for 30 minutes at 30°C (Greenberg and Bender, 1994). Run-on transcripts were isolated according to Pasco *et al* (1988). Hybridization of ^{32}P-labelled RNA to cDNA plasmids immobilized on nitrocellulose membrane was carried out under stringent conditions (50% formamide, 6X SSC, 0.6% SDS, 6X Denhardt's without BSA, 0.02% yeast RNA) at 42°C for 3 days. The membrane was washed for 20 minutes each in

two changes of washing solution (0.1X SSC and 0.1% SDS) at 65°C. The membrane was air-dried, taped to Whatman 3MM paper and exposed to X-ray film for 3-7 days at -70°C

2.7 Cell Culture Experiments

2.7.1 Cell Lines and Cell Propagation

The AHH-1 TK+/- is a human B lymphoblastoid cell line isolated from the RPMI 1788 cell line and was spontaneously transformed by Epstein Barr virus (EBV). The cells contain native CYP1A1 activity which is inducible by pretreatment with appropriate inducers. AHH-1 TK+/- cells have been transfected with vectors containing EBV origin of replication and appropriate selections. cHoI cells contain the vector which selects for histidinol resistance. They retain native inducible CYP1A1 activity. h1A1 v2 cells contain human CYP1A1 cDNA in the vector. The cDNA was introduced into the vector as a 1.7 kb *BclI/Bam* HI fragment of nucleotides 83-1779. CYP1A1 expression is under the control of a herpes simplex virus promoter/enhancer.

cHo1 and h1A1 v2 (Gentest Corporation, Woburn, MA) were propagated in RPMI 1640 medium containing 2 mM l-histidinol, supplemented with 9% $^{v}/_{v}$ horse serum and 2 mM l-glutamine. The cells were incubated at 37°C in a 5% CO_2 atmosphere. Seeding and passaging of cells

was as suggested in the Gentest Corporation Procedures 90-1. Cell viability was routinely checked by trypan blue exclusion test.

2.7.1 Cell Treatment

Cells were diluted to 2 x 10^5 cells/ml and incubated for 24 hours. The cells were then induced with DBA (0.1 μM) in DMSO (1 μl/ml) for 24 hours. In cases where the effect of IFN was investigated, IFN-alpha2a (1000 U/ml) was added to the culture simultaneously with the inducer. h1A1 v2 cells were treated with only IFN.

2.7.2 Ethoxyresorufin 0-deethylase Activity

CYP1A1 activity in the cells was measured by 7-ethoxyresorufin 0-deethylation as described by Burke *et al* (1977). Cells (1 x 10^6/ml) were suspended in assay buffer (50 mM Tris·HCl, pH 8.5, 200 mM sucrose, 10 mM $MgCl_2$ and 2 μM ethoxyresorufin) and incubated at 37°C for 10 minutes as described in Gentest Corporation Procedures 90-1. Total cellular protein content was determined by the method of Bradford (1976) using bovine serum albumin as standard.

2.7.3 Western Immunoblot Analysis

The cells were washed by centrifugation in ice-cold PBS at 3,000 rpm for 5 minutes and then lysed in 5 volumes of ice-cold suspension buffer (100 mM NaCl, 10 mM Tris·HCl, pH 7.6, 1 mM EDTA, pH 8.0 and 100 μg/ml phenylmethyl sulfonyl fluoride [PMSF]). The cell lysate was separated by SDS-PAGE (7.5% running gel and 3% stacking gel) under reducing conditions (Sambrook *et al*, 1989). The procedures for transferring proteins to nitrocellulose membrane, immunodetection and quantification of bands were as described in section 2.4.4.

2.7.4 Isolation and Purification of Total RNA

The cells were recovered by centrifugation at 2,000 rpm for 5 minutes, washed in 10 volumes of ice-cold PBS, recentrifuged and resuspended in 10 volumes of RNA extraction buffer (4.0 M guanidine thiocyanate, 100 mM Tris·HCl, pH 7.5 and 1% β-mercaptoethanol). The suspension was then drawn into a sterile hypodermic syringe and expelled through a 23-gauge needle. This procedure was repeated several times until the preparation was no longer viscous. The suspension was mixed with sarcosyl to a final concentration of 0.5% and centrifuged at 3,000 rpm for 10 minutes at room temperature. The supernatant was then layered onto a cushion of 5.7 M CsCl/10 mM EDTA in a polyallomer ultracentrifuge and centrifuged at 40,000 rpm for 18 hours at

After centrifugation, the RNA pellet was recovered, washed and dissolved in water, as described by Sambrook *et al*, (1989). The preparation was then treated with RNAase-free DNAase and stored at -70°C until needed. The procedures for fractionation of RNA by gel electrophoresis and Northern blot analysis were as described previously in sections 2.5.1 and 2.5.2, respectively.

2.8 Production of cDNA Inserts

Plasmid cDNA inserts were prepared by the triton-lysozyme method of Davis *et al* (1986). The inserts include mouse CYP1A1 cDNA corresponding to nucleotides +333 to +1536, rat CYP1A1 cDNA spanning nucleotides +1918 to +2522 and CYP1A2 cDNA corresponding to nucleotides +287 to +1104, a 1.1 kb human β-actin cDNA, a 1.2 kb human glyceraldehyde-3-phosphate dehydrogenase (G3PD) cDNA. The mouse CYP1A1, human β-actin and human G3PD inserts were obtained from American Type Culture Collection (ATCC, Rockville, MD). The rat CYP1A1 and CYP1A2 cDNAs were provided by D.S. Pasco, Maharishi International University, Fairfield, IA. Only the mouse CYP1A1 cDNA insert was excised from the plasmid by digesting with *Pst* I. On a Northern blot, this probe hybridizes to CYP1A1 and CYP1A2 mRNAs (Cribb *et al*, 1994). The rat CYP1A1 and CYP1A2

and CYP1A2 mRNAs (Cribb *et al*, 1994). The rat CYP1A1 and CYP1A2 probes are specific for CYP1A1 and CYP1A2 mRNAs, respectively (Pasco *et al*, 1993).

2.8.1 Oligonucleotide Probes

Synthetic oligonucleotide probes used include a CYP3A1 22-mer of sequence 5'-CGGATAGGGCTGTATGAGATTC-3' (Omiecinski *et al*, 1990) and an 18-nucleotide oligoT probe. The probes were synthesized by Marine Gene Probe Laboratories, Dalhousie University, Halifax, Canada.

2.8.2 Labelling of Probes

The 1.2 kb mouse CYP1A1 cDNA was labelled to high specific activity using a random primers DNA labelling system (GIBCO BRL, Life Technologies Inc., Burlington, Ontario, Canada). This technique is based on the method of Feinberg and Vogelstein (1983). Oligonucleotide probes were end-labelled with T4 polynucleotide kinase according to the manufacturer's protocol.

2.9 Statistical Analysis

Data are presented as mean ± s.e. The unpaired Student's *t* test was used for statistical comparison between different treament groups in whole

animal experiments described in chapters 3 and 5. The paired *t* test was used for comparison between groups in experiments described in chapters 4 and 6. The level of statistical significance was set at $p<0.05$.

3.0 THE EFFECT OF INDUCTION MECHANISM ON INTERFERON MEDIATED DOWNREGULATION OF HEPATIC CYP3A1 IN FEMALE RATS

3.1 Introduction

IFN and IFN inducers are known to depress hepatic cytochrome P450s. Many constitutive enzymes namely CYP2C11 (Morgan and Norman, 1990), CYP2A2 (Craig *et al*, 1990), CYP1A2, CYP2C6 (Stanley *et al*, 1991), CYP2C12 (Morgan, 1991) and CYP2E1 (Sakai *et al*, 1992) and have been shown to be depressed. Except for a modest loss reported for CYP4A1 (Renton and Knickle, 1990), the inducible P450s have been reported to be resistant to the depressant effects of IFN or IFN inducers (Craig *et al*, 1990 and Stanley *et al*, 1991). Based on these observations it has been suggested that P450 induction may provide protection against IFN mediated downregulation of the enzymes. Induction of the enzymes may offer this protection by preventing or overcoming the depressant effects of IFNs. We designed experiments to investigate the role played by the mechanism of P450 induction on IFN mediated depression of P450 isozymes. We examined the effect of the IFNα/β production by polyIC on the induction of CYP3A1 in the female rat caused by pregnenolone 16α-carbonitrile (PCN) and troleandomycin (TAO). TAO is a

macrolide antibiotic which induces CYP3A1 expression by stabilizing the enzyme whereas the antiglucocorticoid PCN increases the rate of transcription of the CYP3A1 gene (Okey, 1991).

3.2 Animal Treatment and Analysis

Female Sprague-Dawley rats (175-200 g) were divided into 3 groups: uninduced (saline with 0.001% Tween 80; N=4), TAO-induced (200 mg/kg in saline with 0.001% Tween 80; N=8) and PCN-induced (300 mg/kg in saline with 0.001% Tween 80; N=8). The animals treated with inducers were divided into two groups, one of which received saline and the other polyIC (10 mg/kg) intraperitoneally at the same time as the inducer. The animals were killed after 24 hours and livers collected for microsome preparation (section 2.3) and RNA isolation (section 2.5). Liver microsomes were analyzed for the total P450 content, erythromycin N-demethylase activity and CYP3A1 apoprotein.

3.2.1 Slot Blot Analysis of Total RNA

The mRNA encoding CYP3A1 was determined by slot blot analysis of the total RNA using a synthetic CYP3A1-specific oligonucleotide probe of sequence 5'-d(CGGATAGGGCTGTATGAGATTC)-3' corresponding to nucleotides 938-

959 of rat CYP3A1 mRNA (Omiecinski *et al*, 1990). On a Northern blot, this probe recognizes a single band of an apparent size of 2.2 kb present in rats treated with PCN. Total RNA was denatured and loaded in 2-fold serial dilutions in 20X SSC (3 M NaCl, 300 mM sodium citrate) onto nitrocellulose membranes using a slot blot apparatus. The membrane was baked, prehybridized for 4 hours in a solution of 6X SSPE (900 mM NaCl, 60 mM NaH_2PO4 and 6 mM Na_2EDTA, pH 7.4) containing 5X Denhardt's solution (Denhardt, 1966), 1% SDS and 0.11 mg/ml sheared salmon sperm DNA at 42°C. Hybridization was conducted overnight with 5 pmol of ^{32}P-labelled probe in the same buffer at 48°C. The membranes were rinsed at room temperature with 2X SSC and washed 2 times (30-minute durations) in 2X SSC at 48°C and then subjected to autoradiography. The membranes were then stripped and total RNA quantitated with the polyT probe (Harley, 1987). Results were normalized to a standard included on each blot and to the quantity of RNA loaded in each slot as determined by the polyT signal.

3.3 Results

Total microsomal P450 content was increased following treatment with the inducers TAO and PCN. A single dose of polyIC significantly decreased P450 levels by 37% in TAO-treated rats and by 36% in PCN-treated rats (Fig

2). PolyIC did not depress the induction of erythromycin N-demethylation significantly in PCN-treated rats (7.6 ± 2.9 versus 1.1 ± 0.5; p=0.069) (Fig 3). Erythromycin N-demethylase activity was not significantly different from zero in control and TAO-treated rats. Western immunoblot analysis of CYP3A1 apoprotein demonstrated that TAO and PCN induced CYP3A1 apoprotein levels (Fig 4 & 5). PolyIC administration significantly depressed the induction of apoprotein by 84% in TAO-treated animals and 73% in PCN-treated animals (Fig 4 & 5). The depression of CYP3A1 induction by polyIC was accompanied by a decrease in CYP3A1 mRNA levels (Fig. 6 & 7). PolyIC significantly depressed the induction of CYP3A1 mRNA by 52% in PCN-treated rats (Fig 6 & 7). In rats induced with TAO, CYP3A1 mRNA was increased slightly above control (1.4 ± 0.28 AU versus 0.74 ± 0.18 AU; p=0.1). PolyIC depressed this slightly induced mRNA level (0.99 ± 0.31 AU).

Figure 2

Effect of PolyIC on the induction of total P450.

Total P450 was induced by a 24-hour administration of TAO (200 mg/kg) or PCN (300 mg/kg). PolyIC (10 mg/kg) administered simultaneously significantly inhibited the induction of total P450 by TAO and PCN.

Figure 2

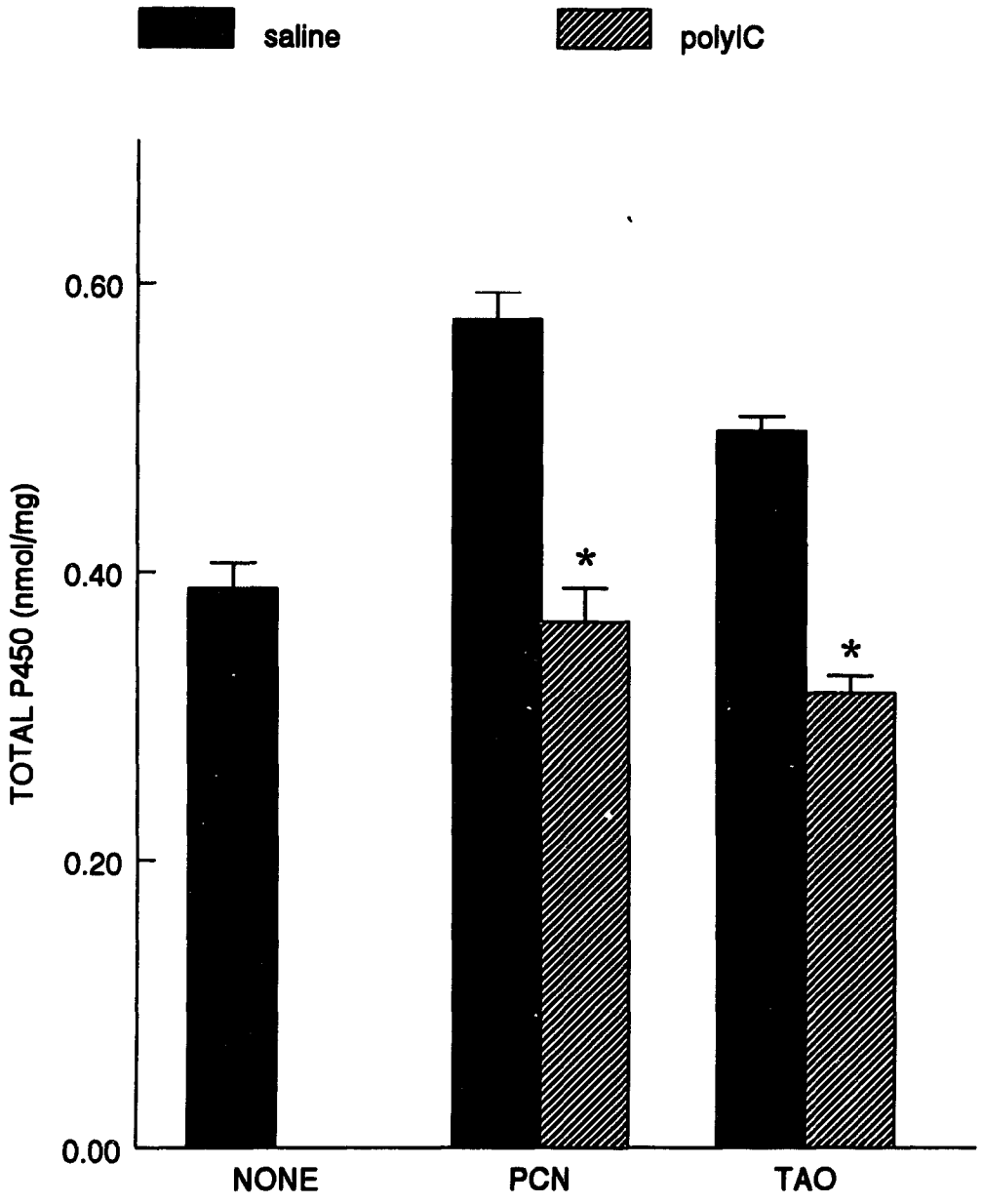


Figure 3

Effect of polyIC on erythromycin N-demethylase activity. Simultaneous administration of polyIC inhibited erythromycin N-demethylation in PCN-treated rats (86%) but this was not statistically significant. Erythromycin N-demethylase activity could not be detected in microsomes prepared from TAO-treated rats.

Figure 3

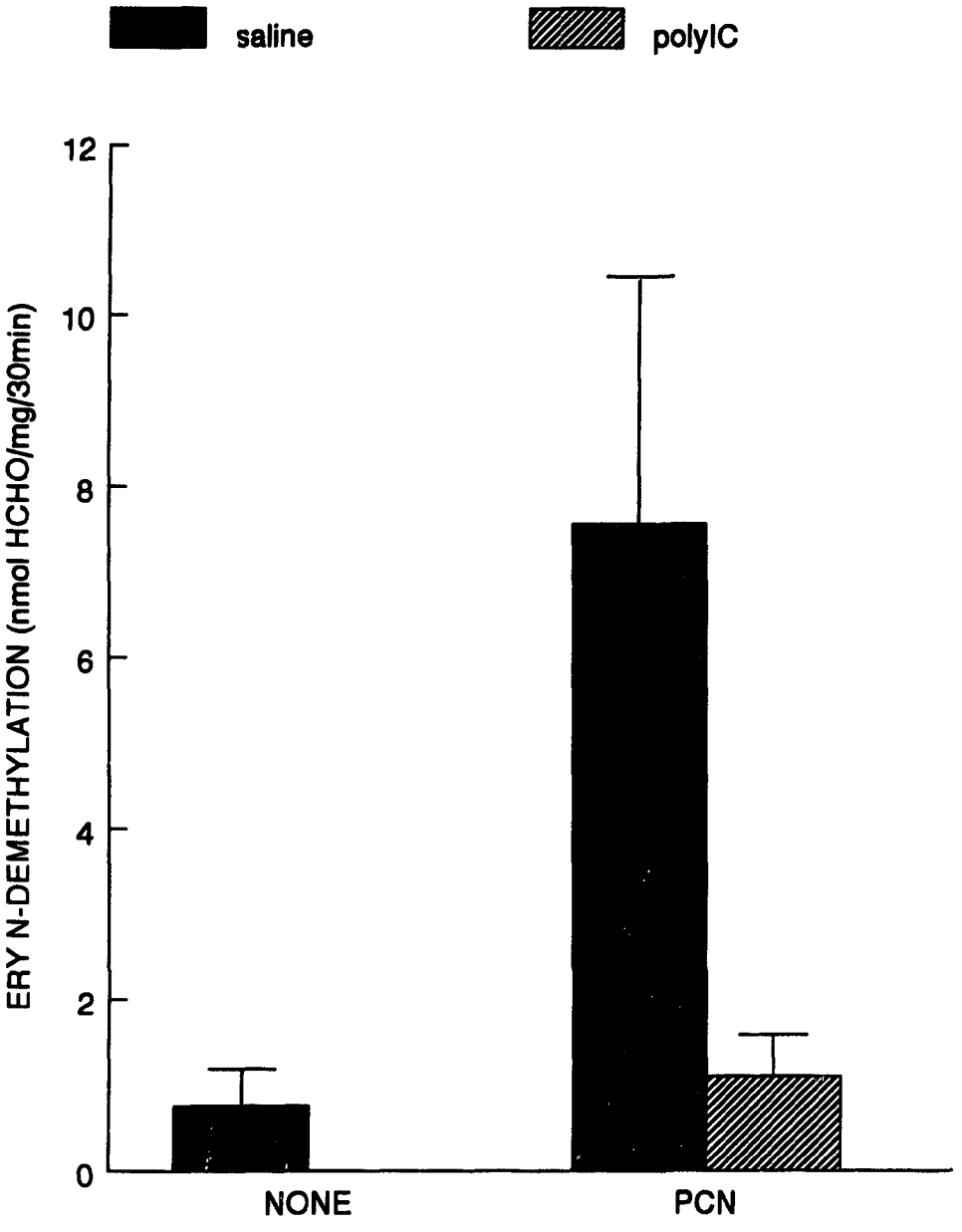

saline
polyIC
ERY N-DEMETHYLATION (nmol HCHO/mg/30min)
12
10
8
6
4
2
0
NONE
PCN

Figure 4

Immunochemical quantitation of CYP3A1 inhibition by polyIC. Measurement of CYP3A1 apoprotein levels showed that PCN and TAO induced the apoprotein. Administration of polyIC significantly inhibited the induction of CYP3A1 apoprotein by both TAO and PCN.

Figure 4

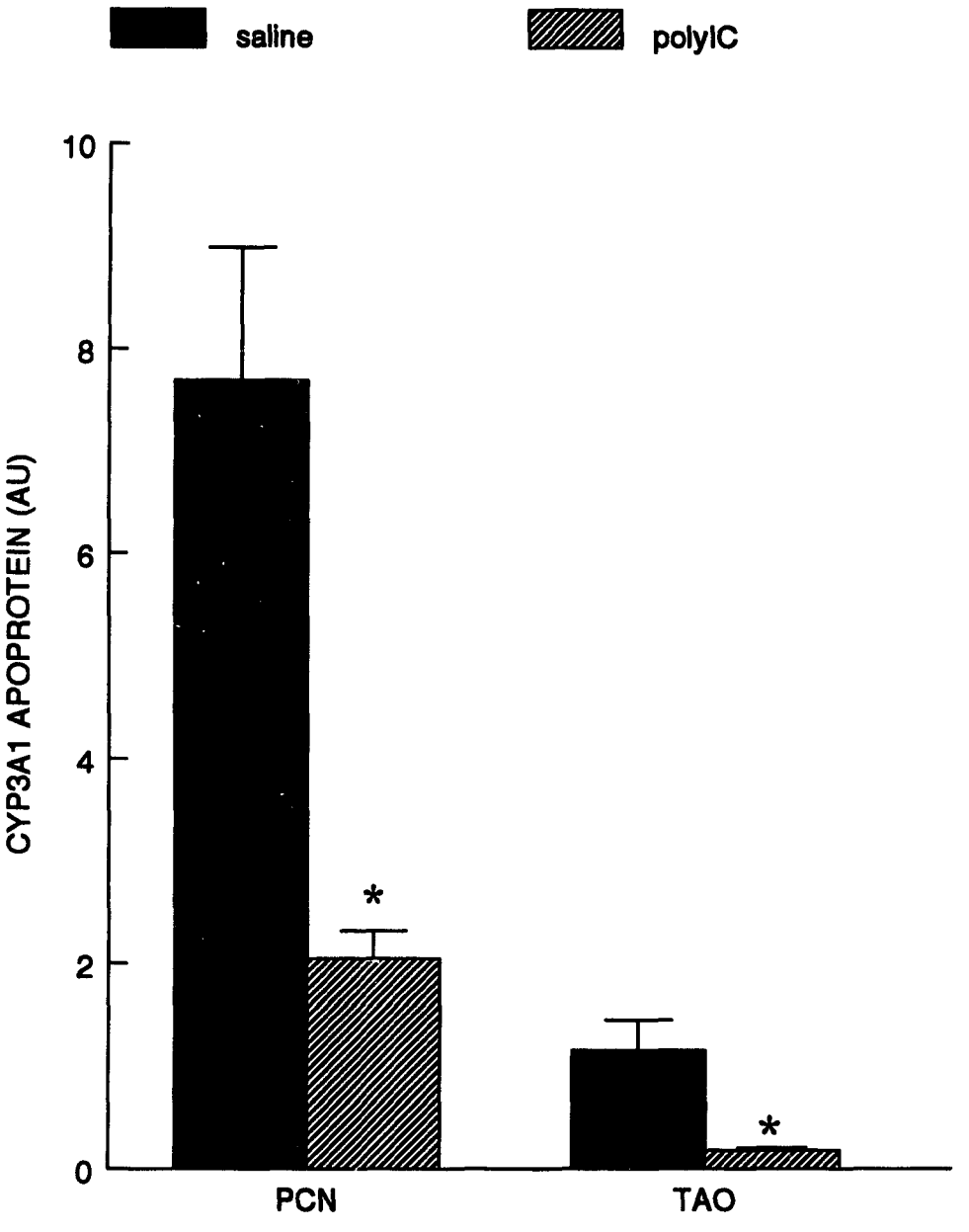
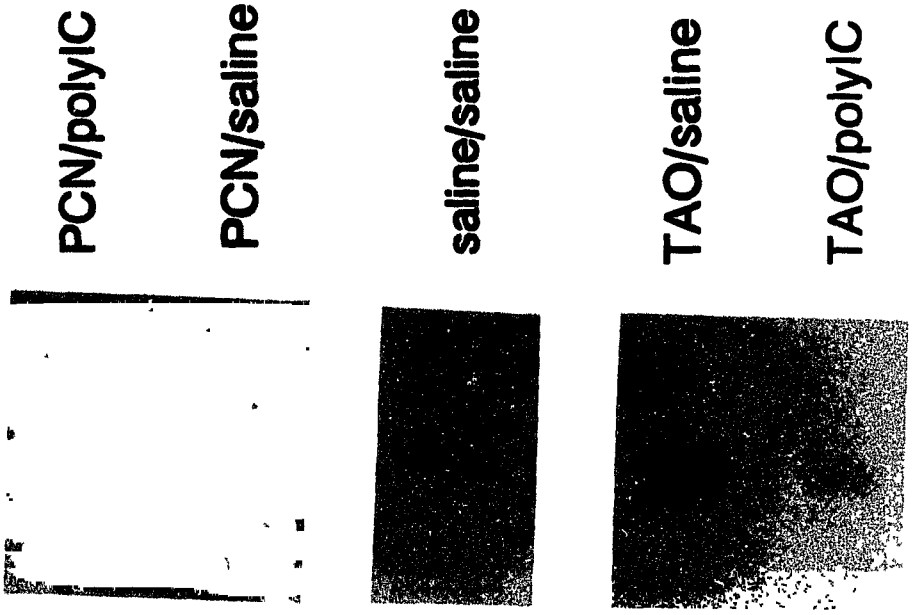


Figure 5



Immunoblots obtained with a polyclonal antirat CYP3A1 antibody. 20 μg of microsomal protein was loaded per lane and electrophoresed on 7.5% SDS-polyacrylamide gel. PolyIC inhibited the induction of CYP3A1 apoprotein by TAO and PCN.

Figure 6

Quantitation of CYP3A1 mRNA by slot blot analysis.

Results are expressed as arbitrary units of CYP3A1 mRNA corrected for the amount of mRNA loaded in the slot as demonstrated by a polyT oligonucleotide probe. PolyIC significantly inhibited the induction of CYP3A1 mRNA by PCN. The slight induction of CYP3A1 mRNA caused by TAO was also inhibited by polyIC administration.

Figure 6

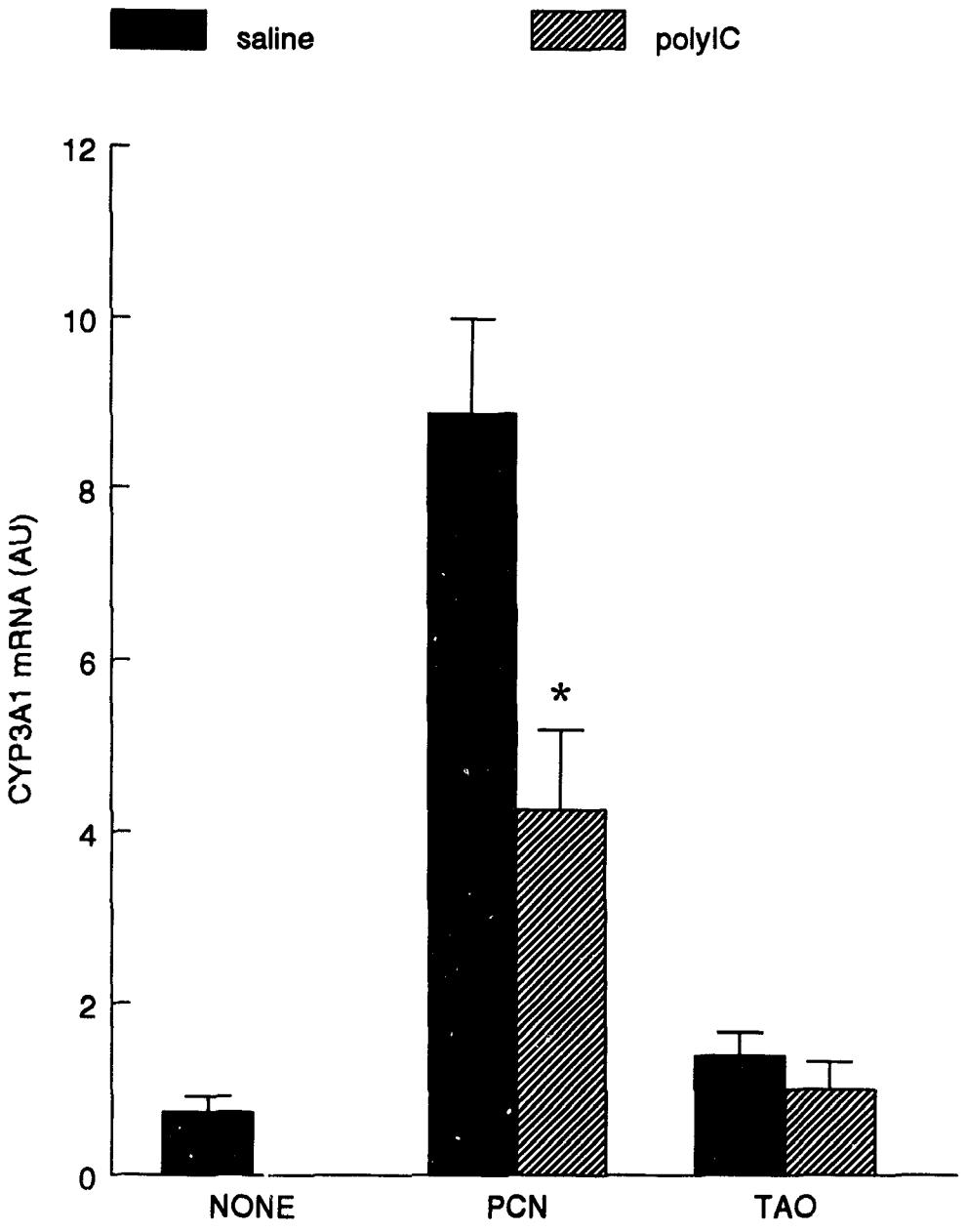
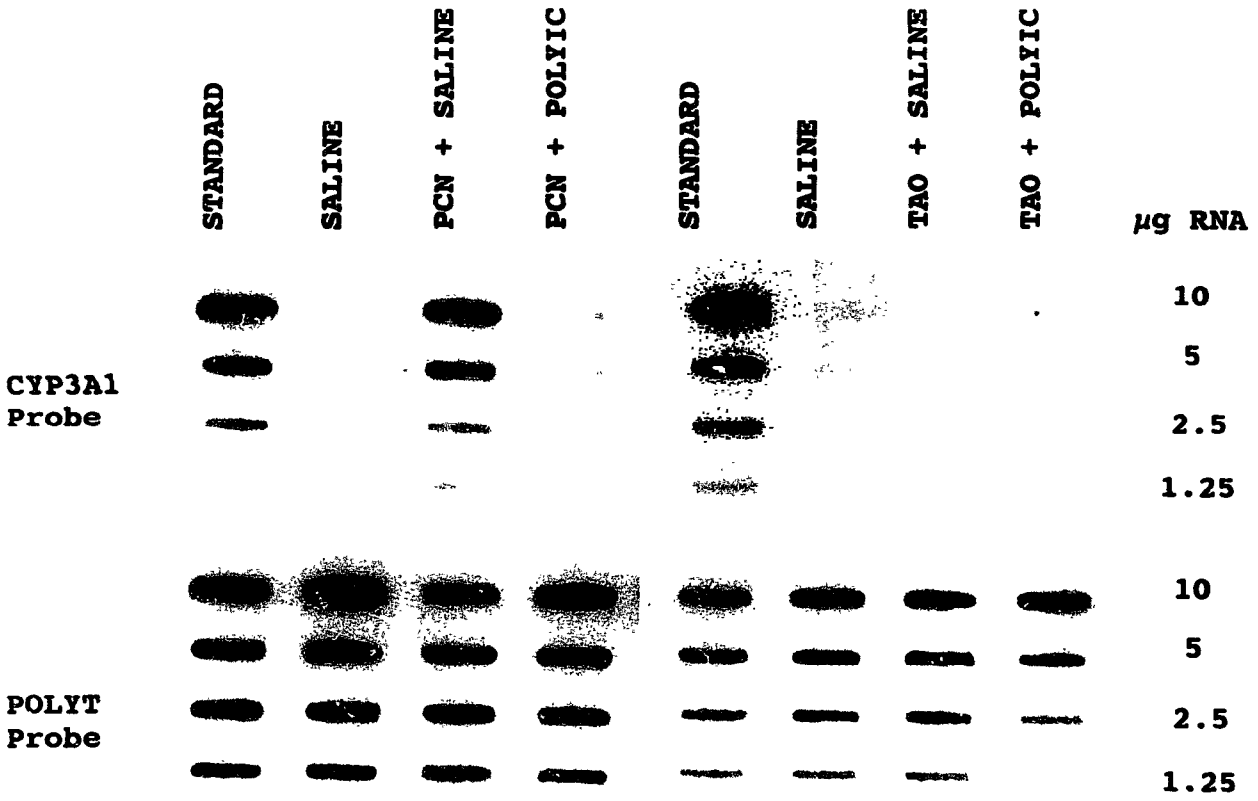


Figure 7

Slot blot analysis of CYP3A1 mRNA.

Representative slot blots obtained by probing the same blot with the CYP3A1 oligonucleotide (top) and the polyT oligonucleotide (bottom). PolyIC inhibited the induction of CYP3A1 mRNA without affecting the total RNA as shown by the polyT probe. TAO caused a slight induction of CYP3A1 mRNA and this was inhibited by polyIC.

Figure 7


STANDARD
SALINE
PCN + SALINE
PCN + POLYIC
STANDARD
SALINE
TAO + SALINE
TAO + POLYIC
µg RNA
CYP3A1 Probe
10
5
2.5
1.25
POLYT Probe
10
5
2.5
1.25

4.0 INTERFERON MEDIATED CHANGES IN THE EXPRESSION OF CYP1A1 IN HUMAN B LYMPHOBLASTOID (AHH-1 TK+/-) CELLS

4.1 Introduction

In addition to the depression of constitutive P450 enzymes by IFN and IFN inducers (Craig *et al*, 1990; Morgan and Norman, 1990; Morgan, 1991; Stanley *et al*, 1991) recent studies conducted in our laboratories suggest that IFN also downregulates the expression of inducible P450s. Even though the exact mechanism by which IFN mediates its effects is not known, there is evidence for the requirement of the *de novo* synthesis of an unknown intermediate protein (Moochhala and Renton, 1991). Several reports have shown that P450 synthesis is depressed as a result of a pretranslational event which leads to loss of the mRNA encoding P450 apoproteins (Craig *et al*, 1990; Morgan, 1990; Morgan and Norman, 1990; Renton and Knickle, 1990; Stanley *et al*, 1991; Armstrong and Renton, 1993; Cribb *et al*, 1994). This pretranslational mechanism has also been shown to be independent of induction processes (Cribb *et al*, 1994; Anari *et al*, 1995; described in chapter 3). Although a pretranslational mechanism has been implicated, whether IFN affects gene transcription and/or mRNA stability is not known. We used two lymphoblastoid cell lines to investigate the effect of IFN on inducible and

transfected CYP1A1.

AHH TK+/- are human B lymphoblastoid cells which contain low native CYP1A1 activity (Davies *et al*, 1989; Crespi *et al*, 1989; Crespi, 1991; Crespi *et al*, 1993; Penman *et al*, 1994). The enzyme activity is inducible by pretreatment with polycyclic aromatic hydrocarbon inducers such as dibenz(*a,h*)anthracene, β-naphthoflavone and 3-methylcholanthrene (Crespi 1991). The mechanism of CYP1A1 induction in AHH-1 TK+/- cells is presumed to be through the cytosolic *Ah* receptor as described for other lymphoblastoid cell lines (Waithe *et al*, 1991). The cells contain adequate levels of P450 oxidoreductase and cytochrome *b5* to support good functional expression of P450s (Crespi, 1991). AHH-1 TK+/- cells have been transfected to express extrachromosomal vector-derived human cDNAs. In transfected cells the low basal activity has been found not to interfere with measurements of enzyme activity. The transfected cell line, h1A1 v2, contains human CYP1A1 cDNA in the extrachromosomal vector. The cDNA was introduced into the vector as a 1.7 kb *Bcl I/Bam* HI fragment containing nucleotides 83 to 1779. The expression of CYP1A1 in h1A1 v2 cells is under the control of a herpes simplex virus promoter/enhancer (Crespi *et al*, 1990; Penman *et al*, 1994) rather than the normal endogenous cellular control mechanisms which regulate CYP1A1 expression through its promoter. In the transfected cell only

regulate CYP1A1 expression through its promoter. In the transfected cell only mRNA stability can be downregulated by cellular mechanisms but in the cells expressing native cytochrome P450, both mRNA stability and transcription can be affected. Thus, downregulation of CYP1A1 mRNA in the cHoI cell line but not the h1A1 v2 would implicate an effect on transcriptional control while depression of CYP1A1 mRNA in both cell lines would implicate an effect on mRNA stability.

4.2 Cell Treatment and Analysis

Two groups containing five plates of each cell type were diluted to $2x10^5$ cells/ml, twenty-four hours later, the cHoI cells were treated simultaneously with DBA (0.1 μM) in DMSO (1 μl/ml) and IFN-alpha2a (1000 U/ml) in RPMI 1640 medium. The h1A1 v2 cells were treated with IFN-alpha2a (1000 U/ml) alone. Control cHoI cells were treated with DBA and RPMI 1640 medium while h1A1 v2 cells were treated with the medium alone. The cells were harvested for analysis twenty-four hours after treatment. The cells were analyzed for ethoxyresorufin O-deethylase activity and CYP1A1 apoprotein as described in sections 2.7.2 and 2.7.3, respectively. The dose of IFN used in this study was based on preliminary work showing this dose was required to produce a change in enzyme activity. This is consistent with

1982) and is not likely to adversely affect the cells (Adams *et al*, 1975).

4.2.1 Northern Blot Analysis of RNA

Isolation of total RNA, gel electrophoresis and Northern transfer were performed as previously described in section 2.7.5. Prehybridization and hybridization were performed as described by Christou *et al* (1990) using a [α-^{32}P]dCTP-labelled 1.2 kb cDNA insert of the mouse CYP1A1 mRNA. The probe corresponds to nucleotides 333 to 1536 relative to the 5' end. The amount of RNA loaded per lane was verified by methylene blue staining of the membrane (Sambrook *et al*, 1989).

4.3 Results

CYP1A1 inducibility in cHoI cells was tested by treating the cells with DBA (0.1 μM)in DMSO (1 μl/ml). At this concentration DBA caused a 5-fold increase in EROD activity (0.685 ± 0.099 versus 0.143 ± 0.048 pmol resorufin/mg protein, p=0.007) (Fig 8).

CYP1A1 activity, apoprotein and mRNA were determined and expressed as percentage increase or decrease in comparison to the control for that experiment. EROD activity in IFN-treated cells is 93% (92.78 ± 3.54, p=0.14) and 122% (121.98 ± 2.38, p=0.002) of control in cHoI and h1A1

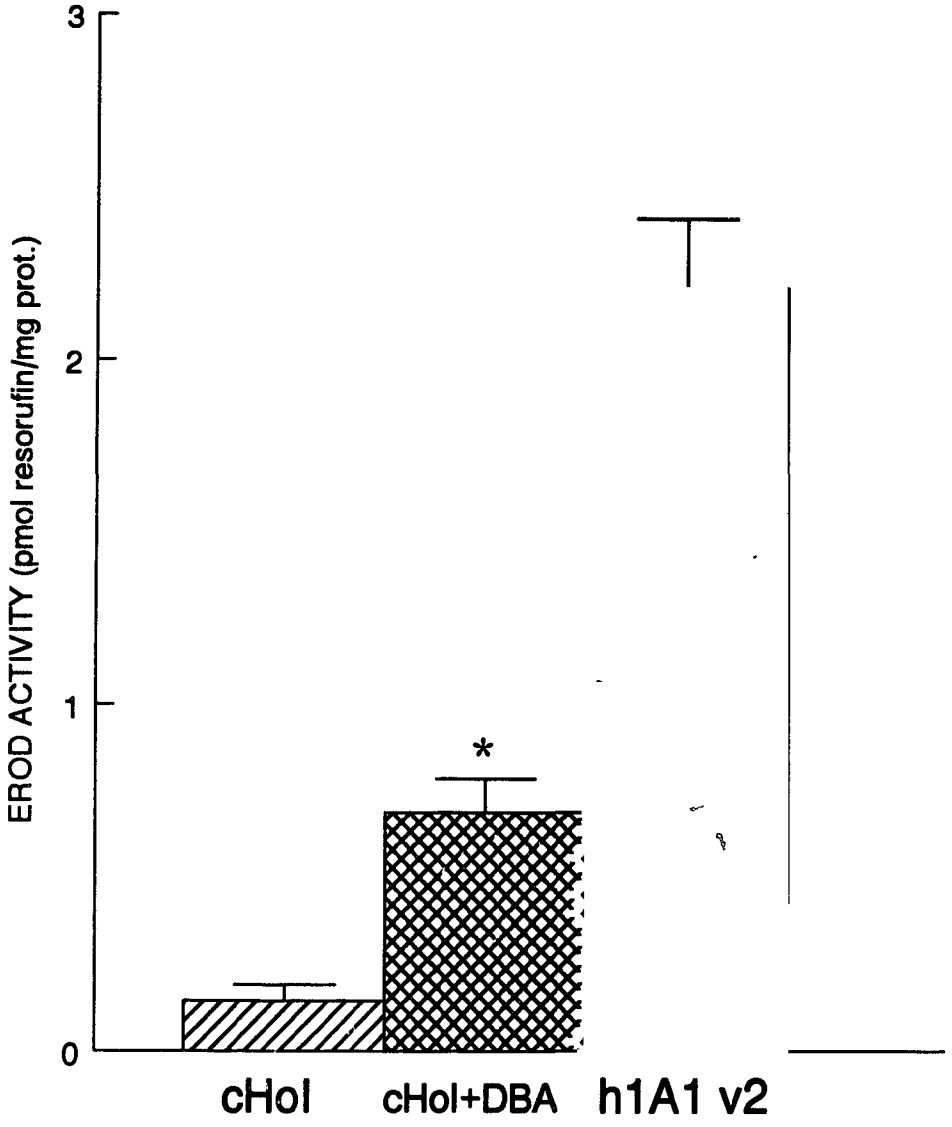
p=0.14) and 122% (121.98 ± 2.38, p=0.002) of control in cHoI and h1A1 v2 cells, respectively (Fig 9). IFN treatment depressed CYP1A1 apoprotein in cHoI cells by 55% (45.17 ± 11.71% of control; p=0.042). In h1A1 v2 cells CYP1A1 apoprotein was increased by 47% (146.73 ± 2.08% of control; p=0.002) (Fig 9 & 10). Northern blot hybridization of mRNA obtained from three pooled cell cultures showed that in agreement with apoprotein levels, CYP1A1 mRNA levels were depressed by 76% in cHoI cells and increased by 208% in h1A1 v2 cells (Fig 9 & 11).

Figure 8

Induction of CYP1A1 activity in cHoI cells

Treatment of cHoI cells with DBA for 24 hours resulted in a statistically significant (5-fold) induction of CYP1A1 activity as determined by ethoxyresorufin O-deethylation.

Figure 8


EROD ACTIVITY (pmol resorufin/mg prot.)
3
2
1
0
*
cHol
cHol+DBA
h1A1 v2

Effect of IFN-alpha2a on CYP1A1 activity, apoprotein and mRNA in cHoI and h1A1 v2 cells.

cHoI cells were treated simultaneously with DBA (0.1 μM) and IFN (1000 U/ml) while h1A1 v2 cells were treated with IFN (1000 U/ml) alone. Human IFN-alpha2a increased CYP1A1-mediated EROD activity by 22% in h1A1 v2 cells (65.75±6.58 versus 80.21 ± 8.58 pmol resorufin/mg protein/min; p=0.004) but had little effect in cHoI cells (10.64 ± 1.53 versus 9.96 ± 1.65 pmol resorufin/mg protein/min; p=0.143). EROD activity represents the mean of % control ± s.e. from five experiments with five culture plates (except for one cHoI control experiment which had four plates) per experiment. IFN treatment depressed CYP1A1 apoprotein in cHoI cells by 55% (45.17 ± 11.71% of control; p=0.042). In h1A1 v2 cells CYP1A1 apoprotein was increased by 47% (146.73 ± 2.08% of control; p=0.002). CYP1A1 apoprotein represents the mean of % control ± s.e. of three experiments. Northern blot hybridization of mRNA obtained from three pooled cell cultures showed that CYP1A1 mRNA levels were depressed by 76% in cHoI cells and increased by 208% in h1A1 v2 cells.

Figure 9

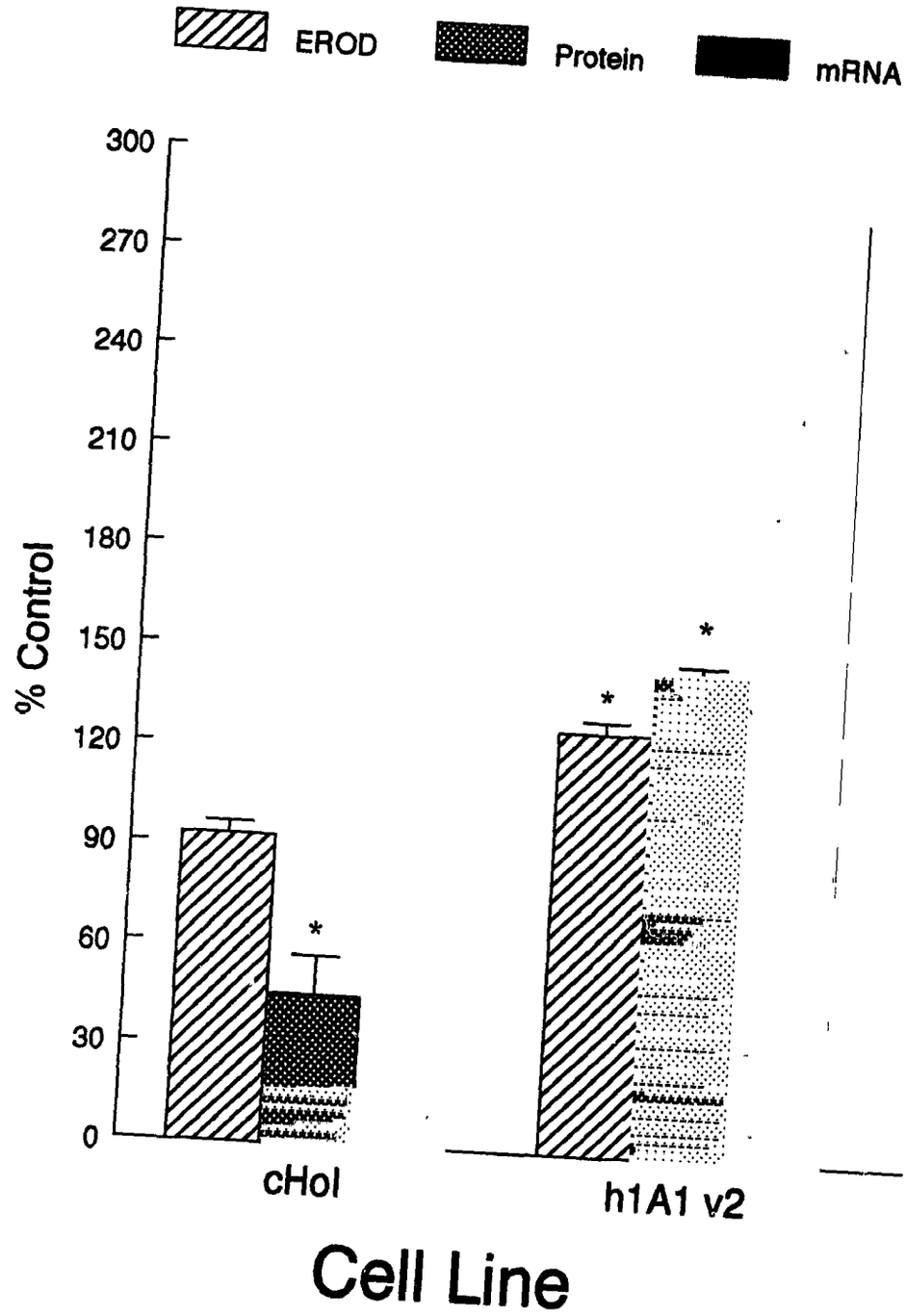
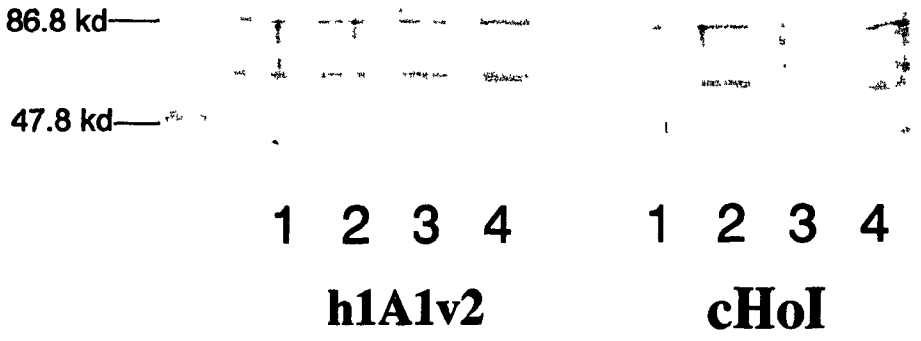
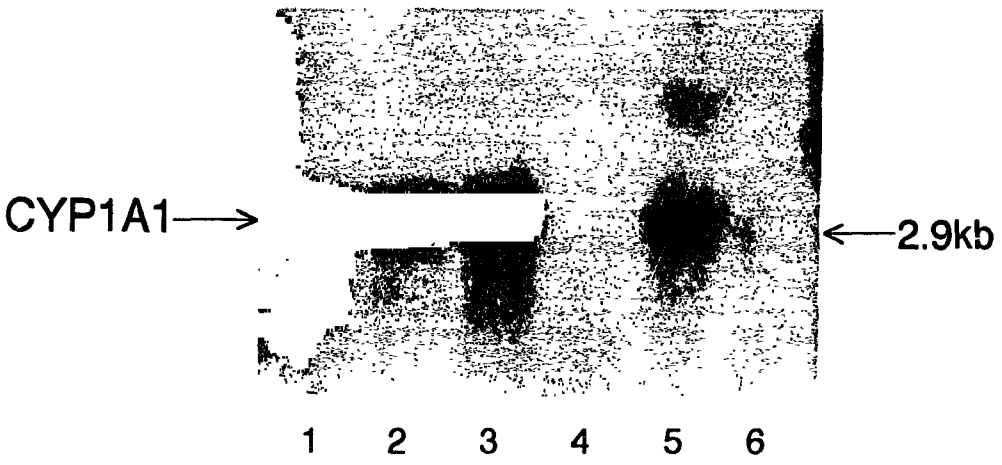

EROD
Protein
mRNA
300
270
240
210
180
150
120
90
60
30
0
% Control
*
*
*
cHol
h1A1 v2
Cell Line

Figure 10



Immunoblots obtained with polyclonal antirat CYP1A1 antibody. Representative Western immunoblots of induced CYP1A1 in cHoI and constitutive CYP1A1 in h1A1 v2 cells. Cell lysate containing approximately 10 μg of protein was loaded per lane from h1A1 v2 cells treated with culture medium (Lanes 1 & 3) and IFN (Lanes 2 & 4), and cHoI cells treated with DBA+IFN (Lanes 1 & 3) and DBA alone (Lanes 2 & 4) and. IFN depressed CYP1A1 apoprotein in cHoI cells. In h1A1 v2 cells IFN increased CYP1A1 apoprotein.

Figure 11

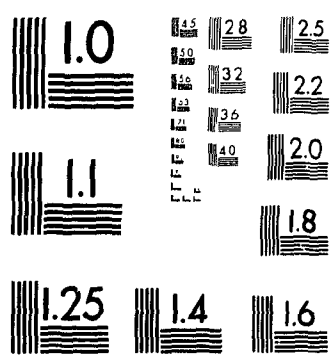


Northern blot obtained by probing with mouse CYP1A1 cDNA. Thirty micrograms of total RNA was loaded per lane and hybridization performed using a mouse CYP1A1 cDNA insert. The probe detected a 2.9 kb. mRNA corresponding to the human CYP1A1 mRNA. Lanes 1 & 3 were loaded with RNA from h1A1 v2 cells treated with IFN. Lane 2 contains RNA from control cells treated with RPMI medium. Lanes 4 & 6 were loaded with RNA from cHoI cells treated with DBA+IFN. Lane 5 was loaded with RNA from cHoI cells treated with DBA.

5.0 INTERFERON α/β INDUCTION ACCELERATES THE LOSS OF CYP1A1 AND CYP1A2 mRNA IN BNF-TREATED RATS

5.1 Introduction

IFN mediated downregulation of P450 enzymes occurs through a pretranslational mechanism which is independent of the mechanism of enzyme induction (described in chapter 3; Cribb *et al*, 1994; Anari *et al*, 1995) and requires the *de novo* synthesis of an unknown intermediate protein (Moochhala and Renton, 1991). Studies we conducted in lymphoblastoid cells (described in chapter 4) suggested that IFN depressed CYP1A1 mRNA by inhibiting the transcription of the gene. There is evidence from nuclear run-on assays that some cytokines regulate P450 activity at the level of gene transcription (Wright and Morgan, 1990; Barker *et al*, 1992; Fakuda and Sassa, 1994) while others do not (Wright and Morgan, 1990; Barker *et al*, 1992). The studies taken together suggest that immune modulators can affect regulation of P450 gene expression at both the transcriptional and posttranscriptional levels. The turnover of mRNA plays a significant role, together with mRNA transcription and processing, in determining the expression of genes in eukaryotes (Belasco and Brawerman, 1993). There is evidence that mRNA stability is determined by the presence or absence of discrete sequences which



act as cleavage sites for exo- and endonucleases. The physiological relevance of controlling of gene expression at the level of the message is exemplified by the rapid turnover of immediate early genes and cytokine mRNAs (Belasco and Brawerman, 1993). The experiments described in this chapter were designed to assess the effect of IFNα/β production on the degradation rates of CYP1A1 and CYP1A2 mRNA levels after transcriptional inhibition with actinomycin D. We used Northern blot analysis to determine the initial cleavage of the mRNA and slot blot analysis to determine the conversion of mRNA transcripts to fragments that cannot bind to the membrane or form RNA-DNA duplex with the probe (Belasco and Brawerman, 1993).

5.2 Animal Treatment and Analysis

Three groups of male Sprague-Dawley rats (200-225 g) animals were used: uninduced (saline N=20), induced (BNF 60 mg/kg in olive oil; N=20) and induced and immune-stimulated (BNF 60 mg/kg in olive oil and polyIC 10 mg/kg in saline; N=20). Six hours following treatment, four animals form each group were killed by CO_2 asphyxiation and liver samples collected for RNA isolation. The remaining animals were divided into two groups, six hours later, one group received saline and the other actimomycin D (1 mg/kg). Four animals from each treatment group were killed at the time of treatment. The

animals from each treatment group were killed at the time of treatment. The remaining animals were killed twelve hours later. Northern blot analysis of total RNA was performed using a CYP1A1 cDNA probe as described previously. The amount of RNA present in each lane after transfer to the membrane was verified by methylene blue staining. Slot blot hybridization of RNA was also performed using the cDNA probe. The total RNA loaded in each slot was quantitated with the polyT probe (Harley, 1987) and the results normalized to a standard included on each blot and to the quantity of RNA loaded in each slot as determined by the polyT signal.

5.3 Results

The treatment of animals with BNF for 12 hours significantly induced CYP1A mRNA as determined by slot blot analysis (0.35 ± 0.04 versus 1.46 ± 0.11 AU; p=0.00007) (Fig 12 & 13). Concomitant administration of polyIC with BNF significantly depressed mRNA levels by 23% (1.46 ± 0.11 versus 1.12 ± 0.06; p=0.033) (Fig 12 & 13).

Twelve hours after transcriptional inhibition by actinomycin, the levels of CYP1A1 mRNA were significantly lowered in rats treated with BNF and rats treated with BNF and polyIC (Fig 14). The treatment of animals with polyIC resulted in a greater loss CYP1A1 mRNA compared to CYP1A2 mRNA during

Northern blotting demonstrated that polyIC enhanced the cleavage of both CYP1A1 and 1A2 mRNAs 12 hours after transcriptional inhibition (Fig 14). Quantitation of CYP1A mRNA levels 12 hours after treatment with actinomycin D by slot blot analysis demonstrated a significant loss of mRNA in rats treated with BNF alone (1.46 ± 0.15 versus 1.04 ± 0.09; p=0.034) and rats treated with BNF and polyIC treated rats (1.50 ± 0.04 versus 0.63 ± 0.03; p=0.00003) (Fig 15 & 16). The loss of mRNA in polyIC treated rats was greater (58%) compared to the loss (28%) in BNF treated rats. Twelve hours after inhibition of transcription, the loss of CYP1A mRNA is significantly different in animals treated with BNF and those treated with BNF and polyIC (1.04 ± 0.09 versus 0.63 ± 0.03 p=0.015) (Fig 15 & 16). In saline treated rats no difference was observed between animals treated with actinomycin and their respective controls (0.26 ± 0.04 versus 0.32 ± 0.02; p=0.24) (Fig 15 & 16). The slot blot analysis showed that following transcriptional blockade the IFN inducer, polyIC, increased the rate at which induced CYP1A mRNA was converted to fragments that cannot bind the membrane or form RNA-DNA duplex with the CYP1A1 cDNA.

Figure 12

Effect of polyIC on CYP1A mRNA induction

The effect of polyIC on hepatic CYP1A mRNA induction was determined by slot blot analysis. Results are expressed as arbitrary units (AU) of CYP1A mRNA corrected for the amount of mRNA loaded in the slots as demonstrated by a polyT oligonucleotide probe. PolyIC (10 mg/kg) administered simultaneously with the inducer significantly depressed CYP1A mRNA level.

Figure 12

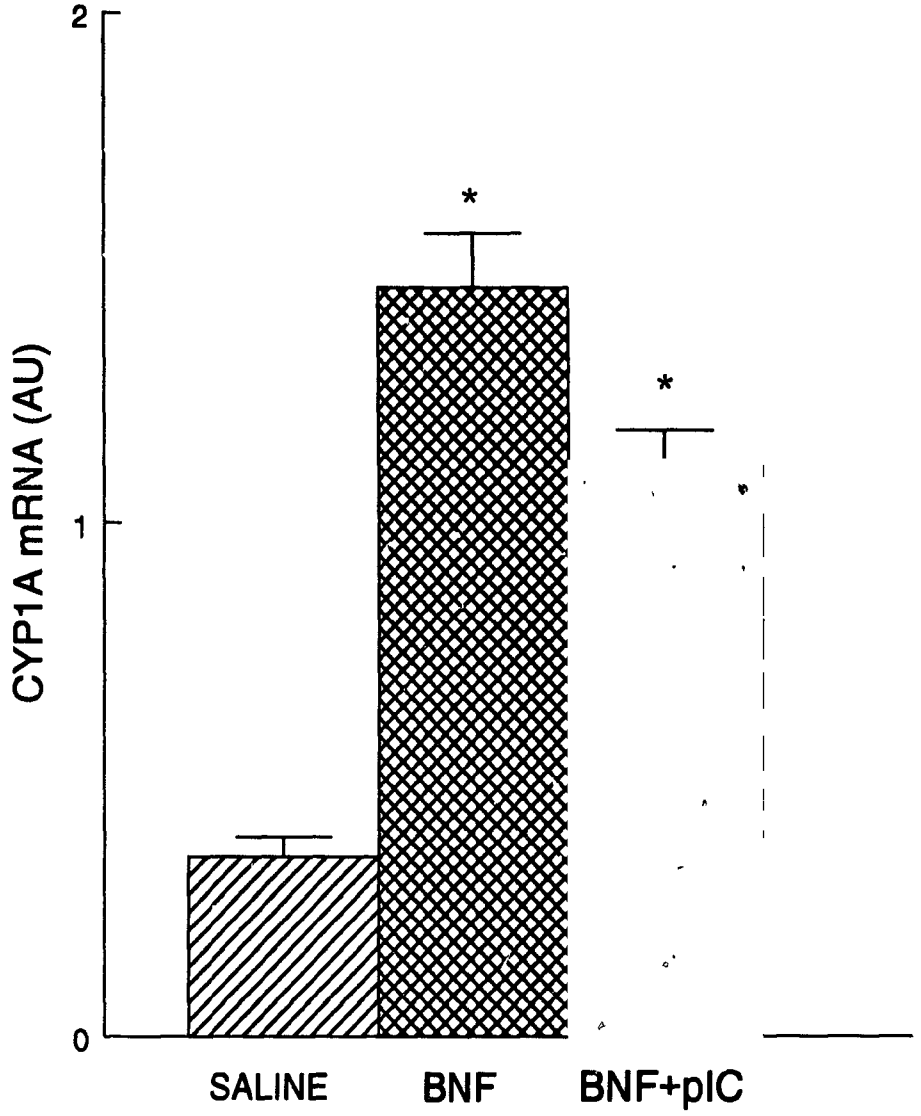
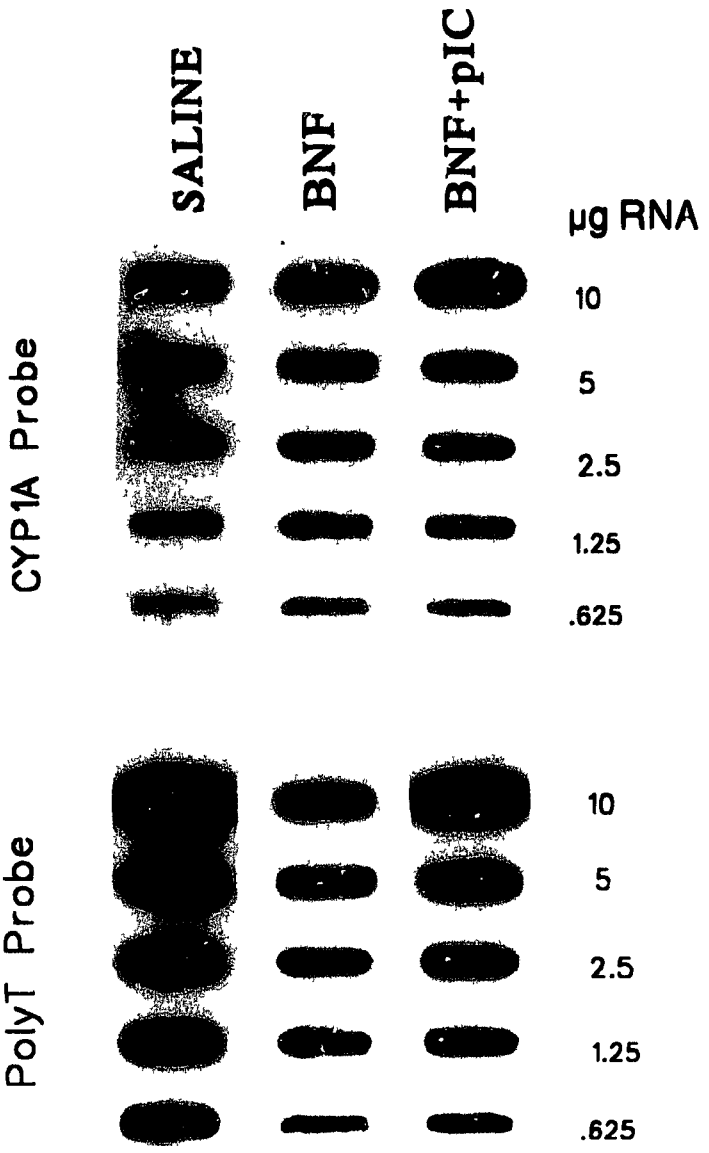


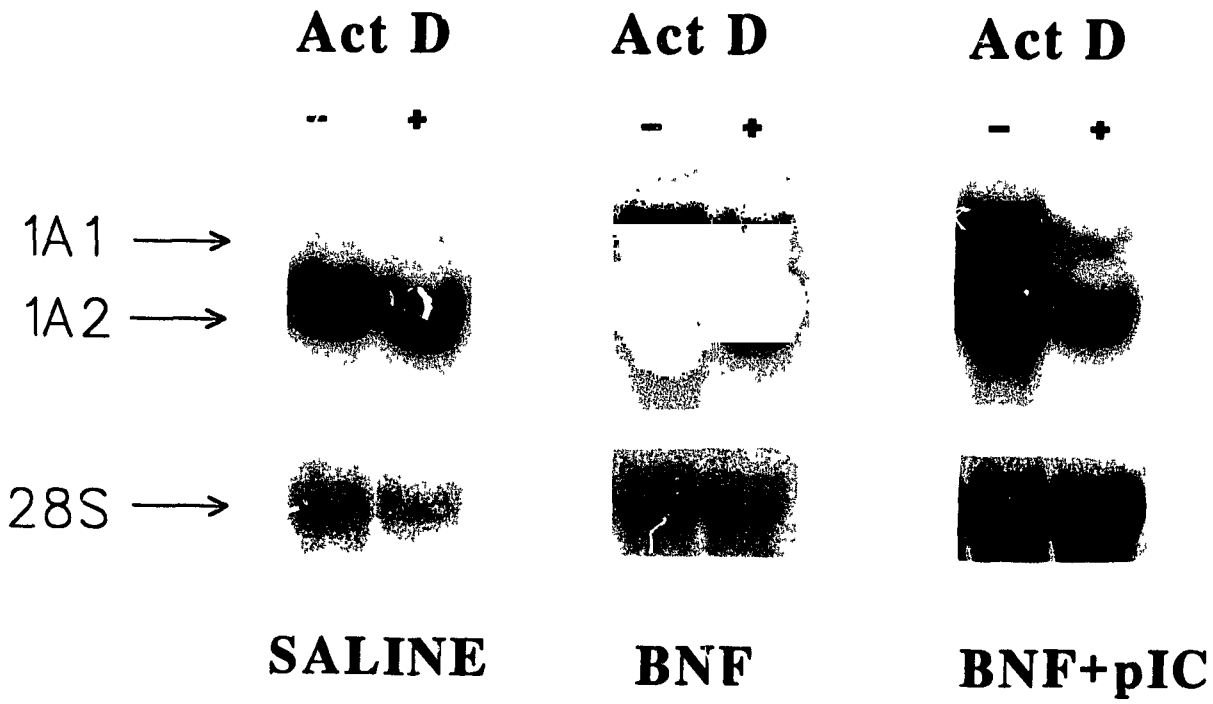
Figure 13



Slot blots obtained by probing with CYP1A1 cDNA

Representati. e slot blots obtained by probing the same blot with a mouse CYP1A1 cDNA insert (top) and the polyT oligonucleoti de probe (bottom).

Figure 14



Northern blot analysis of hepatic CYP1A1 and 1A2 mRNA after transcription blockade.

Total RNA (8 μg) was loaded in each lane and filters hybridized with a mouse CYP1A1 cDNA insert. Methylene blue staining of the filter was used to show the relative amounts loaded in each lane. Simultaneous administration of polyIC with BNF significantly accelerated the loss both CYP1A1 and 1A2 mRNA. (-) indicates no treatment and (+) treatment with actinomycin D.

Figure 15

Quantitation of CYP1A mRNA after transcription blockade.

Results are expressed as arbitrary units (AU) of CYP1A mRNA that is able to bind to filter and form RNA-DNA duplex. Amount of mRNA in each slot was corrected for by using a polyT oligonucleotide probe.

Figure 15

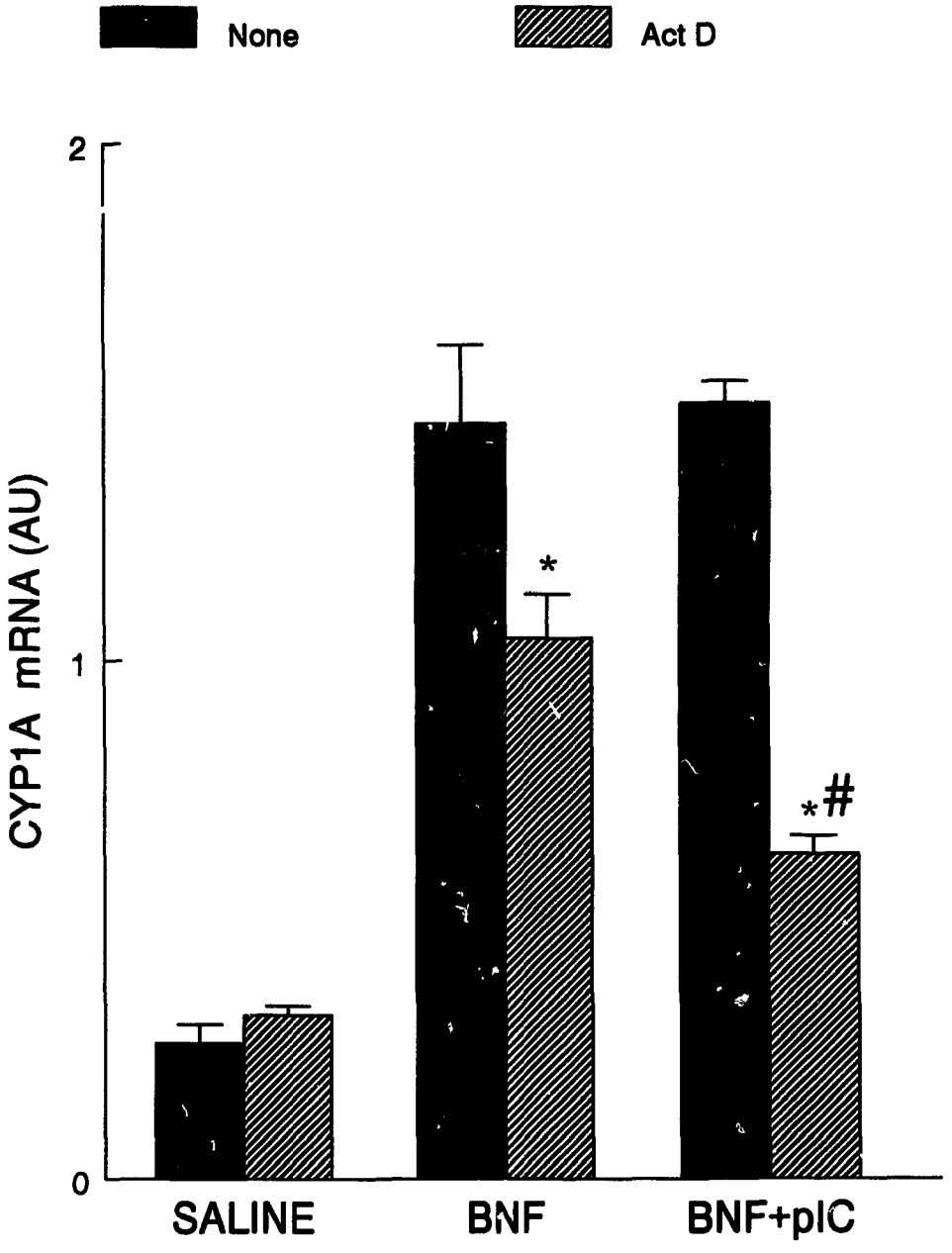
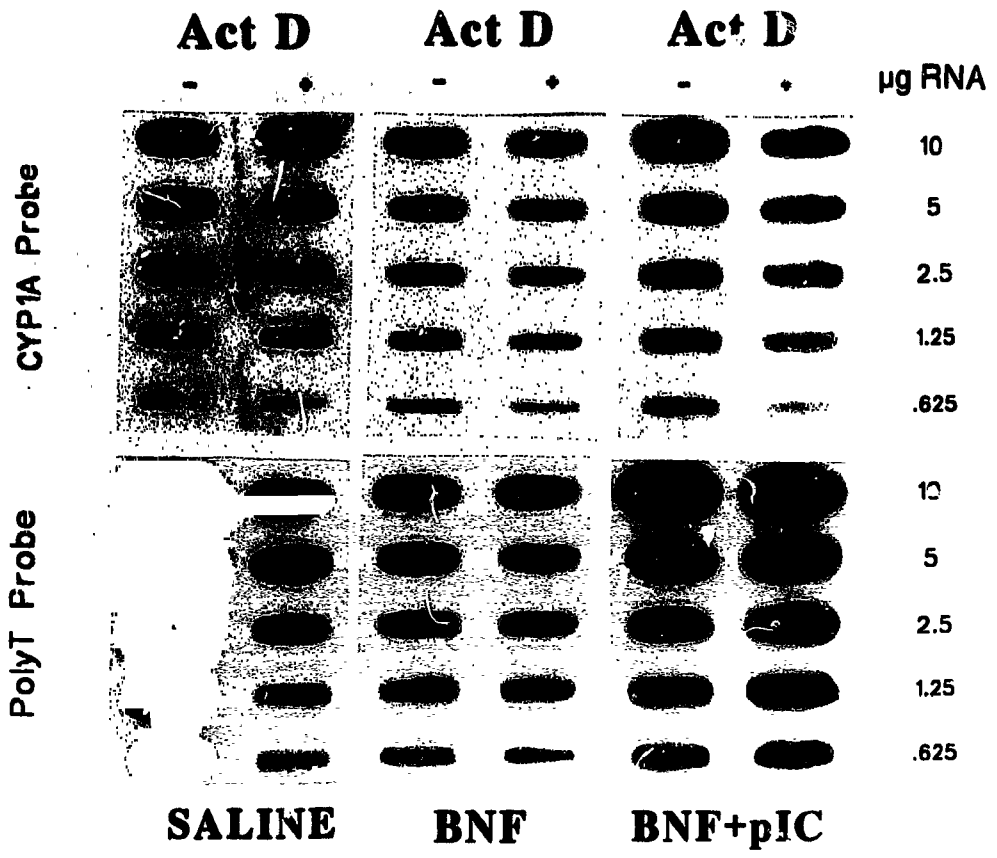


Figure 16



Slot blot analysis of CYP1A mRNA after transcription blockade

Representative slot blots obtained by probing the same blot with a mouse CYP1A1 cDNA insert (top) and the polyT oligonucleotide probe (bottom). (-) indicates no treatment and (+) treatment with actinomycin D.

6.0 INTERFERON-α/β INDUCTION ALTERS THE RATE OF TRANSCRIPTION OF CYP1A GENES

6.1 Introduction

Nuclear run-on transcription assays have been used to measure the relative transcription rates of only a few cytochrome P450s following treatment with immune stimulants. Barker *et al* (1992) showed that IL-1 but not IL-6 and TGF-β suppressed the transcription rates of CYP1A1 and CYP1A2 genes in isolated rat hepatocytes. Recently, Fakuda and Sassa, (1994) demonstrated that IL-6 regulated the expression of CYP1A1 in human HepG2 hepatoma cells at the transcriptional level by a mechanism that appeared to involve heme oxygenase induction. The rate of transcription of CYP2C11 but not CYP2C12 was also reported to be depressed by inflammation induced with endotoxin or turpentine (Wright and Morgan, 1990). Recent studies we conducted with transfected lymphoblastoid cells suggest that IFN may mediate the downregulation of CYP1A1 mRNA through gene transcription. To further characterize the pretranslational mechanisms involved in IFN mediated depression of P450s we performed nuclear run-on transcription assays with hepatic nuclei isolated from rats. This assay enabled us to measure directly the relative rates of transcription of the CYP1A genes following immune

stimulation with polyIC. Our results showed that IFNα/β production decreased the rates of transcription of the CYP1A1 and CYP1A2 genes.

6.2 Animal Treatment and Analysis

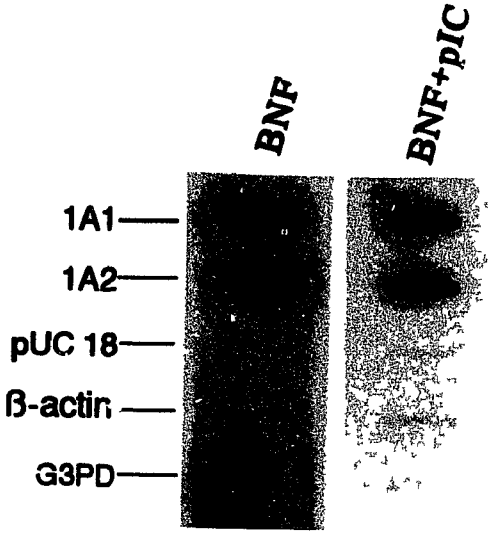
Two groups of male Sprague-Dawley rats (175-200 g) were used: induced (BNF 60 mg/kg in olive oil; N=4) and induced and immune-stimulated (BNF 60 mg/kg in olive oil and polyIC 10 mg/kg in saline; N=4). Twenty-four hours later, the animals were killed and the livers harvested. Nuclei were isolated as previously described (section 2.6). Rat CYP1A1 and CYP1A2 cDNAs corresponding to nucleotides +1918 to +2522 and +287 to +1104, respectively, a 1.1 kb human β-actin cDNA, a 1.2 kb human G3PD cDNA and pUC 18 vector were bound to nitrocellulose membrane (described in section 2.6.1) and run-on transcription analysis performed as described in section 2.6.2.

6.3 Results

Run-on transcripts isolated from nuclei of rats treated with BNF and polyIC showed a decrease in the relative transcription rates of CYP1A1 and CYP1A2 genes in comparison with transcripts obtained from nuclei of rats treated with BNF alone (Fig 17). Quantitation by densitometry showed that polyIC

decreased the rate of transcription of CYP1A1 and CYP1A2 genes by 22% (78 ± 3.21% of control; p=0.021) and 26% (74 ± 3.51% of control; p=0.018), respectively. The results are the mean of % control ± s.e. of three measurements. The specificity of this decrease is confirmed by the very low signal obtained for nonspecific binding to the pUC18 cDNA. Immune stimulation by polyIC also lowered the rate of transcription of the β-actin gene confirming the depression of actin mRNA by the IFN inducer reported by Morgan and Norman (1990).

Figure 17



Effect of polyIC on the transcription rates of CYP1A genes.

RNA transcripts initiated *in vivo* were labelled by incubating with ^{32}P(GTP). Nascent RNA transcripts were isolated and hybridized to the designated cDNA probes which were immobilized on nitrocellulose membranes. After hybridization, the blots were washed, subjected to autoradiography and quantitated by densitometry.

7.0 DISCUSSION, SUMMARY AND CONCLUSIONS

7.1 Enzyme Induction and P450 Downregulation by IFN

IFN and IFN inducers depress the hepatic expression of many constitutive and inducible P450 isozymes (Craig *et al*, 1990; Morgan and Norman, 1990; Morgan, 1990; Renton and Knickle, 1990; Stanley *et al*, 1991; Cribb *et al*, 1994; Anari *et al*, 1995; described in chapter 3). Although the exact mechanism of IFN mediated depression of P450s is not known, many investigators have provided evidence to show that the loss is the result of a pretranslational step leading to the depression of mRNA encoding P450 apoproteins (Renton and Knickle, 1990; Craig *et al*, 1990; Morgan and Norman, 1990; Morgan, 1991 and Stanley *et al*, 1991). Previous reports have suggested that induction of P450s may render them resistant to the depressant effects of IFN or IFN inducers (Craig *et al*, 1990; Stanley *et al*, 1991). In the present experiments, however, polyIC depressed the induction of CYP3A1 by both PCN and TAO as demonstrated by erythromycin N-demethylation and Western blot analysis. The depression occurred at a pretranslational step as evidenced by the depression of CYP3A1 mRNA. CYP3A1 activity induced by TAO could not be detected by erythromycin N-demethylation owing to the low level of the enzyme induced by the 24-hour treatment protocol, however,

Western blot analysis clearly demonstrated downregulation of the apoprotein. Craig *et al*, (1990) found no depression of androstene 6β-hydroxylation when they coadministered PCN and recombinant IFNγ to female rats. This lack of agreement with our results may arise from the differences in half-lives of recombinant rat IFNγ and IFNα/β produced in response to polyIC. Recombinant IFNs have short half-lives *in vivo*, whereas polyIC produces a large and sustained increase in serum IFN. The rapid clearance of recombinant IFNs may lead to differences in response when comparisons are made between the effect of IFN itself and IFN inducers. Mannering *et al* (1986) showed that pure recombinant mouse IFNα, IFNβ and IFNγ depress the P450 systems; however, when IFNγ was used, three intraperitoneal injections given 3 hours apart were required suggesting that IFNγ is cleared more rapidly.

In this study two inducers which act by different mechanisms were employed. TAO acts primarily by stabilizing the CYP3A1 enzyme while PCN acts by increasing the transcription rate (Okey, 1990). PolyIC administration exerted similar effects on the mRNA and apoprotein from TAO-treated and PCN-treated rats suggesting that IFNs exert an effect which is independent of the mechanism of P450 induction. Since mRNA is required for P450 apoprotein synthesis, changes in mRNA levels, whether at the level of transcription or mRNA stability would be expected to lead to proportional changes in P450

apoprotein. It remains to be determined whether IFN or IFN induction affects transcription or mRNA stability. It is clear, however, that induction of cytochrome P450 does not invariably protect against the pretranslational depression of apoprotein synthesis mediated by IFNs. These studies showed that CYP3A1 was depressed by the IFNα/β produced in response to polyIC. IFN appeared to act at a pretranslational step that is independent of the induction process *per se*. There are several reports in the literature to support our contention that cytochrome P450 induction does not protect from or confer resistance to depression of P450s by cytokines (Table 2). The table illustrates the diversity of inducible P450 isozymes that are susceptible to downregulation by IFN, IFN inducers and other cytokines. Anari *et al* (1995) attributed the lack of effect of IFNα on inducible CYP1A1 and CYP2B10 in CBA mouse to a low depressant potential of the IFN treatment regimen employed. Stanley *et al* (1991) did not document depression of constitutive P450s in the same animals suggesting that the 3-day IFN treatment regimen used was ineffective in downregulating both inducible and constitutive P450s. The prototype inducers used and the isozymes investigated in the studies summarized in the table shows the wide spectrum of cytochrome P450 induction mechanisms including, gene transcription (1A1/1A2, 3A1,

Table 2

Depression of inducible P450 isozymes by IFN and other cytokines.

Cytochrome P450	Inducer	Reference
1A1/1A2	β-naphthoflavone	Cribb *et al*; Anari *et al*;
	benzo(a)pyrene	Abdel-Razzak *et al;*
	3-methylcholanthrene	Clark *et al*
2B1/2B2, 2B10	phenobarbital	Anari *et al*; Clark *et al*;
		Abdel-Razzak *et al*
2C6/2C7	phenobarbital	Abdel-Razzak *et al*
2E1	pyridine	Cribb *et al*; Anari *et al*
		Abdel-Razzak *et al*
3A1/3A2	phenobarbital, PCN, TA0	chapter 3, Abdel-
		Razzak *et al*
4A1	clofibrate	Renton and Knickle

Based on Renton and Knickle (1990), Cribb *et al* (1994), Abdel-Razzak *et al* (1994, 1995), Anari *et al* (1995), Clark *et al* (1995) and results reported in chapter 3 of this thesis.

2B1/2B2/2B10 and 4A1), processing and mRNA stabilization (1A1/1A2 and 2B1/2B2) and posttranslational stabilization (2E1 and 3A1) which did not protect the respective isozymes from cytokine mediated downregulation. This supports our view that susceptibility of P450 isozymes to IFN and other cytokines is independent of the mechanism of P450 induction. Anari *et al*, (1995) reported susceptibility of highly induced levels of CYP1A1/1A2, CYP2B10 and CYP2E1 to polyIC treatment in Swiss albino mice but resistance of the orthologous enzymes in Sprague-Dawley rats suggesting that the level of induction and species may be the main determinants of susceptibility of P450 enzymes to IFNs and other cytokines.

7.2 Role of the Nucleus in P450 Downregulation by IFN

Recent studies we conducted suggest there are no P450 enzyme families with inherent resistance to downregulation by IFN and P450 induction does not invariably confer resistance or protect against depression by IFN (Cribb *et al*, 1994; described in chapter 3). In this study we demonstrated that inducible native CYP1A1 in lymphoblastoid cells was susceptible to downregulation by IFN confirming that the depression is not limited to hepatic cytochrome P450s alone. The loss of enzyme was seen at the level of mRNA and apoprotein. EROD activity in cHoI cells was unaffected by IFN

treatment. This unexpected result could be due to limiting amounts of NADPH-cytochrome P450 reductase in the cells. Cytochrome P450s and the P450 reductase are known to interact by forming a 1:1 functional complex for the transfer of the first reducing equivalent involved in catalysis (Miwa and Lu, 1984). In the presence of a limited amount of reductase the EROD activity measured in induced cells may be much lower than the maximum that is attainable and not a true reflection of the apoprotein levels. There is evidence that P450 may exist in a large excess over reductase (10:1 to 25:1) following treatment with inducers (Peterson *et al*, 1976). There is also evidence that limiting NADPH-cytochrome P450 reductase resulted in low rate of EROD activity in cardiac microsomes prepared from guinea-pigs induced with β-naphthoflavone (McCallum *et al*, 1992). In IFN-treated cells it is possible for EROD measurements to remain unchanged in the presence of limited reductase activity even though CYP1A1 apoprotein levels were depressed.

In cells expressing CYP1A1 extrachromosomally, IFN caused an up-regulation of enzyme activity, mRNA and apoprotein. These results clearly demonstrate differences in response of CYP1A1 present in the two cell lines to IFN treatment. The pretranslational downregulation of CYP1A1 in cHoI cells and the lack of a similar downregulation in h1A1 v2 cells indicates the importance of nuclear mechanisms in IFN mediated depression of inducible

P450s. A possible mechanism for P450 mRNA downregulation by IFN is inhibition of transcription by nuclear factors which interact with the promoter region of the P450 gene. Since the transfected cDNA in h1A1 v2 lacks the CYP1A1 promoter, it would not be expected to be depressed by nuclear factors specific for P450 downregulation. IFN mediated downregulation of P450 could also result from an increased degradation of mRNA via the dsRNA-dependent oligoadenylate synthetase-RNase L pathway. RNase L cleaves single-stranded RNA after UA, UC, UG, UU residues at the 3' end (Floyd-Smith *et al*, 1981). IFN mediated induction of oligoadenylate synthetase-RNase L pathway has been described in lymphoblastoid cells (Krishnan and Baglioni, 1980; Tomita *et al*, 1982; Rosenblum, 1991; Salehzada *et al*, 1993) and has been shown to lead to increased degradation of c-myc mRNA in Daudi lymphoblastoid (Dani *et al*, 1985) and human colon carcinoma cells (Chatterjee and Savarese, 1992). Although the vector-derived cDNA in the h1A1 v2 has a truncated 3' end, it still contains many RNase L cleavage sites and mRNA degradation due to RNase L would be expected to occur in both cell lines. The depression of CYP1A1 in cHo1 cells in contrast to the increase in h1A1 v2 cells makes mRNA degradation an unlikely mechanism for native CYP1A1 downregulation. The mechanism by which IFN induced the vector-derived P450 is not well understood. However, it is clear that the increase in

P450 in h1A1 v2 cells is not due to a nonspecific interaction between IFN and the vector because cHoI cells have the same vector without the cDNA. IFN could act directly on the herpes simplex virus promoter/enhancer or bind to a region of the coding sequence to produce an upregulation. IFN could also act posttranscriptionally to upregulate P450 expression. Friedman *et al* (1984) reported that IFN upregulated mRNA in neuroblastoma cells through a posttranscriptional mechanism suggesting that the increase in CYP1A1 mRNA could result from posttranscriptional events. There are two reports of induction of P450 enzyme activity in hepatocytes by IFN (Nebert and Friedman, 1973; Renton *et al*, 1978). Currently, it is not known whether transcriptional or posttranscriptional mechanisms are responsible for the upregulation of the extrachromosomal P450 in h1A1 v2 cells.

Barker *et al* (1992) reported the suppression of the transcription rates of CYP1A1 and CYP1A2 genes by IL-1 but not IL-6 or TGFβ. The effect of IL-6 is not well defined because it has also been demonstrated to downregulate CYP1A1 at the transcriptional level by Fakuda and Sassa (1994). The mechanism of IL-6 mediated transcriptional downregulation of CYP1A1 is thought to involve the induction of heme oxygenase (Fakuda and Sassa, 1994). Transcriptional regulation has also been reported to be involved in the suppression of CYP2C11 but not CYP2C12 by inflammation induced with

endotoxin or turpentine (Wright and Morgan, 1990). Since our data suggested that nuclear mechanisms are involved in CYP1A1 depression by IFN we are proposing that the downregulation of CYP1A1 mRNA occurs at the transcriptional level.

7.3 IFNα/β Induction Accelerates Loss of P450

The experiments we designed to show changes in mRNA levels after blockade of transcription with actinomycin D revealed that polyIC accelerated the loss of CYP1A1 and 1A2 mRNA. These findings are supported by the recent demonstration that insulin downregulated the expression of CYP2B and 2E at the posttranscriptional level (De Waziers *et al*, 1995) suggesting that posttranscriptional regulation of P450 expression is a mechanism by which hormones and immune modulators can modify drug metabolism. Messenger RNA degradation results from the action of RNases which hydrolyze nucleic acid at specific and nonspecific sites. The two types of enzymes involved are exonucleases which remove the 5' cap or the 3' polyA tail and/or endonucleases which cleave the mRNA at specific or nonspecific sites (Brawerman, 1987; Sachs, 1993; Decker and Parker, 1994). Nuclease cleavage sites characterized in mammalian cells include the AU-rich destabilizing sequence found in short-lived mRNAs of cytokines, lymphokines and

immediate early genes (Shaw and Kamen, 1986; Zubiaga *et al*, 1995), the 3' stem-loop on histone mRNA (Levine *et al*, 1987) and the iron-responsive destabilizing element on transferrin receptor mRNA (Casey *et al*, 1988; Mullner and Kuhn, 1988). It has been suggested that sequence elements regulating mRNA decay occur throughout the entire message (Sachs, 1993). There is also evidence that the sequences respond to a wide variety of stimuli and cellular signals (Belasco and Brawerman, 1993; Sachs, 1993). The questions raised by these observations concern the nature of sequence elements present in CYP1A mRNAs and whether or not IFN acts directly or indirectly on these elements to promote mRNA loss. IFN is a known inducer of RNase L, an endonuclease which cleaves single-stranded mRNA after UA, UC, UG, UU residues at the 3' end (Floyd-Smith *et al*, 1981; Salehzada *et al*, 1993). RNase L degrades viral mRNA and can, under certain conditions, promote the decay of cellular mRNAs. The induction of the 2'-5' oligoadenylate synthetase-RNase L pathway by IFN has been described in a wide range of cells including liver cells (Krishnan and Baglioni, 1980; Nilsen *et al*, 1981; Lengyel, 1982; Dani *et al*, 1985; Rosenblum, 1991), coupled with the presence of many RNase L cleavage sites on CYP1A1 and 1A2 mRNA makes degradation by this pathway a likely mechanism. The RNase L pathway was proposed as a likely mechanism for IFN mediated depression of cytochrome P450 mRNAs. To

date, no experiments have been performed to support its involvement, as such the of role RNase L remains speculative. IFN could also accelerate the decay of CYP1A mRNAs by repressing the activity of endogenous RNase inhibitors which protect against random degradation by endoribonucleases. This repressor could be an IFN inducible factor. Evidence for interaction between nucleases and nuclease-inhibitors is provided by Bandypadhyal *et al* (1990) who demonstrated the inhibition of a nuclease associated with mRNA in its nucleoprotein state by a soluble cytoplasmic factor. The induction of such repressor is not known, but considering the fact that IFN induces over thirty proteins many of whose functions are not known (Sen and Lengyel, 1992), it is logical to speculate the induction of a nuclease repressor. Messenger RNAs also contain elements at their termini which protect from exonuclease degradation. In eukaryotes the 5' cap structure, and to an extent, the 3' polyA tail are examples of mRNA stabilizers (Belasco and Brawerman, 1993). Disruption of the protective function of these elements could be a mechanism of mRNA destabilization. Although no such role has been defined for IFN or an IFN-inducible product, there is evidence that deadenylation precedes the decay of many eukaryotic mRNAs (Decker and Parker, 1994) and for a mRNA decapping enzyme in yeast (Belasco and Brawerman, 1993; Sachs, 1993). We observed a greater loss of CYP1A1 mRNA by polyIC in comparison to 1A2

mRNA. The differences in susceptibility could result from the different types of destabilizing sequences which might be present. Since no such sequences have as yet been characterized for the P450s, the extent of their contribution is not known. The mechanisms of induction could explain the greater susceptibility of CYP1A1 to degradation. CYP1A1 and 1A2 mRNAs are induced at the transcriptional and posttranscriptional levels. For CYP1A induction, however, posttranscriptional mechanisms are thought to account largely for mRNA increases (Porter and Coon, 1991) It is possible that posttranscriptional stabilization of CYP1A2 mRNA could confer some resistant against the loss induced by polyIC. A question raised by the differential susceptibility of the constitutive and the inducible isozyme is whether posttranscriptional stabilization is required to maintain adequate levels of the constitutively expressed enzyme in contrast to the inducible form which is not normally expressed.

We have demonstrated that IFN induction accelerates the loss of P450 mRNAs. Our results taken together with other evidence suggest that posttranscriptional degradation of mRNA is involved in the pretranslational downregulation of the P450 enzymes by immune modulators. To further characterize the extent of decay caused by IFN and its inducers, measurement of mRNA half-lives will be required.

7.4 IFN Production Decreases Transcription of CYP1A Genes

The fact that nuclear mechanisms are involved in CYP1A1 downregulation by IFN in transfected lymphoblastoid cells led us to propose that IFN mediates its pretranslational effects at the transcriptional level. We have confirmed these results by directly measuring the rate of transcription of the CYP1A1 and CYP1A2 genes by nuclear run-on assays. The assay essentially follows the rate of elongation of RNA transcripts initiated *in vivo* thereby providing a measure of the level of gene transcription at the time that nuclei were isolated. It has been suggested that elongation of previously initiated transcripts accounts largely for the bulk of radioactivity incorporated into RNA. The role of new chain synthesis in isolated nuclei, however, remains controversial. The estimated rate at which the nascent RNA is elongated is 20% of the *in vivo* rate (Marzluff and Huang, 1985). Run-on transcription analysis of CYP1A genes showed that induction of IFNα/β by polyIC decreased the rate of transcription of both CYP1A1 and CYP1A2 genes. To date, IL-1β and IL-6 are the only cytokines that are known to regulate the expression of CYP1A1 gene at the transcriptional level (Barker *et al*, 1992; Fakuda and Sassa, 1994). Barker *et al* also demonstrated that IL-1 suppressed the rate of transcription of the CYP1A2 gene in isolated rat hepatocytes. The results from these studies taken together with the

results from these studies taken together with the posttranscriptional loss (described in chapter 5) following IFNα/β induction suggest that transcriptional and posttranscriptional mechanisms are involved in IFN mediated downregulation of CYP1A1 and CYP1A2 genes. Our results do not preclude the involvement of other pretranslational mechanisms including mRNA processing and mRNA transport, they only suggest that the pretranslational depression caused by IFN and its inducers may be mediated by a combination of transcriptional and posttranscriptional mechanisms.

7.5 SUMMARY

1. IFNα/β production depressed the hepatic expression of CYP3A1 at a pretranslational step.
2. Induction of CYP3A1 did not protect against IFN mediated downregulation.
3. IFN-alpha2a depressed CYP1A1 mRNA and apoprotein expression with no significant effect on enzyme activity in cHoI cells.
4. IFN-alpha2a upregulated CYP1A1 mRNA, apoprotein and activity in h1A1 v2 cells.
5. Nuclear mechanisms are essential for IFN mediated downregulation of CYP1A1.
6. IFN induction increased the rate of loss of CYP1A1 and CYP1A2 mRNA.
7. Loss of CYP1A1 mRNA was greater than that observed for CYP1A2 implying a differential susceptibility of the induced enzyme to posttranscriptional degradation.
8. IFNα/β induction by polyIC decreased the rates of transcription of the CYP1A1 and CYP1A2 genes.
9. The pretranslational loss of CYP1A1 and CYP1A2 caused by IFN and IFN inducers involves depression of gene transcription.

7.6 CONCLUSIONS

1. IFN mediated downregulation of P450 is not dependent on the mechanism of P450 induction.
2. Both constitutive and inducible P450s are susceptible to IFN mediated downregulation.
3. Transcriptional and posttranscriptional mechanisms are involved in the loss of cytochrome P450s caused by IFN and IFN inducers.

7.7 Proposals for Future Work

1. To investigate the role of RNase L in IFN mediated P450 downregulation.
2. To measure the rate of degradation of CYP1A1 and CYP1A2 mRNA induced by IFN.
3. To characterize the transcriptional effects of IFN on P450 gene expression.
4. To investigate the involvement of posttranscriptional mechanisms such as mRNA processing and mRNA transport.

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