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POTENTIAL MEDIATORS OF THE DOWN-REGULATION OF CYTOCHROME P450 DURING CENTRAL INFLAMMATION

by

Tara Nicholson

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
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August, 2001

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

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External Examiner:

Research Supervisor:

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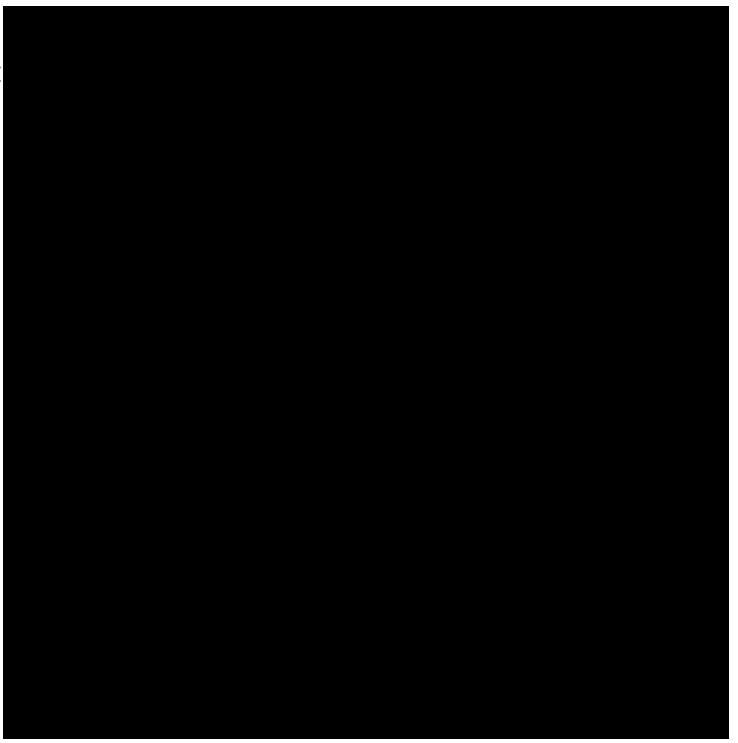

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Tara Nicholson (author)

This thesis is dedicated to my wonderful husband Brian, and to my family who have lovingly encouraged me from the start.

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ABSTRACT

Potential mediators involved in the down-regulation of cytochrome P450 enzymes in the brain and liver during a central inflammatory response induced by the endotoxin, lipopolysaccharide (LPS) were investigated. A role for cytokines as mediators of this depression in cytochrome P450 activity in brain and liver is proposed. An *in vitro* model utilizing cultured astrocytes was used to examine the direct effects of LPS on induced levels of CYP1A activity in these brain-derived cells. Results demonstrated that the acute phase cytokines TNF-α and IL-1β were produced from these cells in response to incubation with LPS and that these same cytokines modulate CYP1A activity in these cells when added exogenously. In addition, substantial levels of nitric oxide were measured in cultures incubated with LPS and blockade of this release partially attenuated the depression in CYP1A activity.

Administration of LPS into the lateral ventricle of the brain was used as an *in vivo* model of central inflammation. CYP1A activity and protein in both brain and liver were depressed in response to this treatment. The administration of the pro-inflammatory cytokines TNF-α, IL-1β, and IFN-γ directly into the lateral ventricle emulated the effects of LPS on CYP1A activity only in the brain. In contrast, these centrally administered cytokines did not produce a concomitant loss of CYP1A activity in the liver.

Significant levels of several cytokines (TNF-α, IL-1β, and IFN-γ) were produced in the serum of animals following i.c.v. LPS administration. This production of peripheral cytokines by LPS could not be mimicked by the administration of IL-1β nor TNF-α by i.c.v. injection. In addition, peripherally administered cytokines, at concentrations similar to those detected in serum following i.c.v. injection of LPS, were shown to decrease cytochrome P450 activity in liver.

These results suggest that induction of cytokines in the brain may play a direct role in the depression of CYP1A activity in the CNS following the administration of LPS into the lateral ventricle. The production of cytokines within the brain does not appear to participate in the signaling process in the brain that leads to the concomitant loss of

CYP1A2 activity in the liver. The subsequent production of cytokines in peripheral tissues does however, appear to play a role in the loss of cytochrome P450 in the liver.

ABBREVIATIONS AND SYMBOLS

AA	arachidonic acid
ACTH	adrenal corticotrophic hormone
AH	aromatic hydrocarbon
AHR	aromatic hydrocarbon receptor
AHRE	aromatic hydrocarbon response element
AID	aromatic hydrocarbon-interacting protein
ANOVA	analysis of variance
ARNT	aromatic hydrocarbon receptor nuclear translocator protein
AP-1	activator protein-1
B cells	bone marrow-derived lymphocytes
βNF	β-naphthoflavone
CNS	central nervous system
cGMP	cyclic guanidine monophosphate
CO	carbon monoxide
COX	cyclooxygenase
CRF	corticotrophin-releasing factor
CSF	cerebral spinal fluid
CYP	cytochrome P450
CZX	chlorzoxazone
DAB	3, 3'-diaminobenzidine tetrahydrochloride
DBA	dibenz[*a*,*h*]anthracene
DEX	dexamethasone
DMEM	Dublecco's modified eagles medium
DMSO	dimethylsulfoxide
EROD	ethoxyresorufin O-deethylase
eNOS	endothelial nitric oxide synthase
FAD	flavin adenine dinucleotide
FADD	Fas-associated death domain
FBS	fetal bovine serum

FITC	fluorescein isothiocyanate
FMN	flavin mononucleotide
GAS	gamma activating site
G-CSF	granulocyte-colony stimulating factor
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte-macrophage-colony stimulating factor
HPA	hypothalamic pituitary adrenal
HPLC	high performance liquid chromatography
hsp	heat-shock protein
ICE	IL-1β converting enzyme
i.c.v.	intracerebroventricular
IFN	interferon
IL	interleukin
IL-1R	interleukin 1 receptor
IL-1ra	interleukin 1 receptor antagonist
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IRF-1	interferon regulatory factor
ISRE	interferon stimulation response element
JAK	Janus kinase
L-Arg	L-arginine
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
3-MC	3-methylcholanthrene
MCP-1	monocyte chemoattractant protein
MHC	major histocompatibility complex
MIP	macrophage inhibitory protein
MS	Multiple Sclerosis
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NGF	nerve growth factor

nNOS	neuronal nitric oxide synthase
NSAID	non-steroidal anti-inflammatory drug
NF-κB	nuclear factor kappa B
NO	nitric oxide
PB	phosphate buffer
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PLA_2	phospholipase A_2
PNTX	pentoxifylline
POD	peroxidase
PolyIC	polyriboinosinic-polyribocytidylic acid
PROD	pentoxyresorufin O de-alkylase
Prot.	protein
RIP	receptor interacting protein
RANTES	regulated on activation, normal T cell expressed and secreted
RT-PCR	reverse transcriptase-polymerase chain reaction
Res.	resorufin
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SNP	sodium nitroprusside
STAT	signal transducer and activator of transcription
T cells	thymus-derived lymphocytes
TGF-β	transforming growth factor-beta
TNF-α	tumour necrosis factor-alpha
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
TRITC	tetramethylrhodamine isothiocyanate

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Drug Metabolism

The body has developed many intricate and interdependent mechanisms for the excretion of foreign compounds. Drug metabolism primarily serves to increase the polarity of an exogenous compound resulting in altered biological activity, and/or an increase in its rate of excretion from the body. Generally, drug metabolism will inactivate a drug, however, this does not always occur and various classes of pro-drugs require metabolic alterations before they become biologically active (Correia, 1995).

Drug metabolism is subdivided in two phases, I and II. Phase I primarily involves oxidative, reductive, and hydrolytic reactions, which alter or add functional groups to these compounds. Phase II reactions involve conjugation of a drug and/or its metabolites to endogenous compounds such as glucuronic acid. These reactions serve to increase the polarity of the compound and thus facilitate its excretion from the body by eliminating its ability to be reabsorbed in the kidneys (Correia, 1995).

Cytochrome P450 enzymes are primarily responsible for carrying out phase I reactions. The majority of these reactions involve the incorporation of an oxygen atom derived from molecular oxygen into the substrate. As well, these enzymes catalyze reactions such as de-halogenation, and the S-, N-, and O- de-alkylations. All of these reactions increase substrate polarity and thus are important in drug elimination (Gibson and Skett, 1986).

1.2 Cytochrome P450

Cytochrome P450s are a superfamily of structurally related, heme-containing enzymes that are primarily responsible for the metabolism of xenobiotics and the synthesis of various endogenous compounds such as eicosanoids, steroids and fatty acids

(Coon et al., 1992). Cytochrome P450 isoforms occur in most biological systems including microorganisms, plants, and animals. Some species specificity does occur, however, many isoforms are closely related in the evolutionary tree and support similar functions in the various species.

1.2.1 History

Evidence for cytochrome P450 existence was first reported in 1958 by two separate researchers, Garfinkel and Klingenberg. Both were working on properties of liver microsomes when they discovered that exposure of reduced microsomes to carbon monoxide (CO) resulted in a characteristic absorption peak at 450 nm (Garfinkel, 1958; Klingenberg, 1958). Subsequent to this discovery Omura and Sato determined the heme containing nature of this "carbon monoxide-binding pigment" and termed it cytochrome P450 (Omura and Sato, 1962). Estabrook, Cooper, and Rosenthal (1963) first uncovered the physiological role of this binding pigment as an oxygen activator and later its role in the hydroxylation and oxidative de-alkylation of drugs.

Certainly the discovery of cytochrome P450 and its role in drug metabolism sparked much interest and investigation into its properties and mode of regulation. While investigating the mechanism of phenobarbital tolerance, Remmer and Merker (1963) discovered that chronic administration of phenobarbital led to a concomitant decrease in the sedative effects of the drug. Indeed, they determined that phenobarbital was capable of stimulating its own metabolism. In a similar vein, work done by Conney et al. (1956) showed that co-administration of 3-methylcholanthrene (3-MC) with the pro-carcinogenic aminoazo dyes led to a decrease in their carcinogenic activity compared to when the dyes

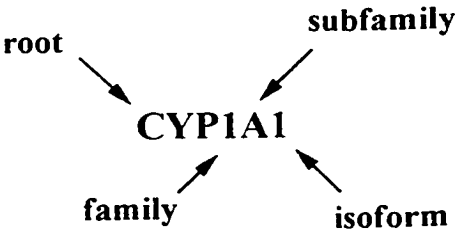
were administered alone. It was found that treatment with 3-MC induced the metabolism of the aminoazo dyes and prevented their carcinogenic activity. These types of experiments showed that cytochrome P450 expression is not static but can be modified by chemical inducers such as phenobarbital and 3-MC.

Originally it was thought that cytochrome P450 was a single enzyme. However, induction studies by Sladek and Mannering (1966) revealed different induction patterns depending on the chemical inducer used. The advent of more sensitive biochemical methods and molecular biology has led to the discovery of a vast array of cytochrome P450 isoforms.

1.2.2 Nomenclature

As alluded to above, the discovery of the vast array of cytochrome P450 isoforms has led to the necessity of a systematic method for naming related isoforms. Prior to 1987, some P450 isoforms carried a multitude of different designations depending on the species in which they were detected and the activity they displayed. For example, the CYP1A1 isoform was also called c, BNF-B, P_1, $P_1$450, MC, and P448, names that gave little information to its activity or genetically related counterparts (Lewis, 1996a). Resulting from the confusion of these types of trivial names, Nebert et al. (1987) proposed a systematic method for naming the cytochrome P450 isoforms. This method was based upon their evolutionary divergence and the amino acid similarity between isoforms. The cytochrome P450 gene was subdivided into families, subfamilies and finally individual isoforms. A cytochrome P450 isoform with a sequence homology greater than 40% is derived from the same gene family while a 59% homology indicates that they are part of

the same subfamily (Gonzalez, 1990). In a similar manner, cytochrome P450 proteins are named using the root 'CYP' to indicate cytochrome P450 followed by the family, subfamily, and individual isoform as for the gene nomenclature. Below is an example of the protein nomenclature:



1.2.3 Evolution

The superfamily of cytochrome P450 enzymes can be divided into two general categories based on their activity and location within the cell. Mitochondrial cytochrome P450s are presumed to have emerged approximately $3x10^9$ years ago and are among the oldest forms of cytochrome P450 in existence. As their name implies, these isoforms reside in the mitochondrial membrane of cells and are highly conserved between species (Gonzalez, 1992). Mitochondrial isoforms participate in the synthesis of a variety of endogenous compounds that are required for basic functions such as cellular membrane integrity and steroid biosynthesis (Gonzalez, 1990). The second category of cytochrome P450 enzymes are the microsomal isoforms that reside in the membrane of the endoplasmic reticulum. These isoforms are thought to have diverged from the mitochondrial forms approximately 900 million years ago. Microsomal isoforms are not as highly conserved owing to their continual divergences to cope with the increasing numbers of chemical/environmental factors to which they are exposed (Gonzalez, 1990).

1.2.4 Regulation

As discussed previously, the expression of cytochrome P450 isoforms does not remain static but can fluctuate dramatically in response to a variety of factors. These fluctuations occur in response to an altered physiological environment and serve to protect the host from harm. In many cases specific isoforms of cytochrome P450 are induced in response to environmental and/or chemical insults. For example, the cytochrome P450 isoform, CYP1A1 is induced in the lung by polycyclic aromatic hydrocarbons in cigarette smoke (Gonzalez, 1992). The mode of regulation that occurs depends upon the particular isoform; in the case of the CYP1A subfamily, expression of both CYP1A1 and CYP1A2 is induced by activation of the aromatic hydrocarbon receptor (Okey et al., 1994). Aromatic hydrocarbons (AH) cross the lipid membrane of cells and interact with the cytosolic aromatic hydrocarbon receptor (AHR). This receptor is maintained in its receptive configuration by hsp90 and the aromatic hydrocarbon-interacting protein (AIP). Binding of the aromatic hydrocarbon to its receptor displaces the inhibitory hsp90 molecule and AIP. The AH-AHR complex enters the nucleus where it heterodimerizes with the aromatic hydrocarbon receptor nuclear translocator protein (ARNT) to generate a DNA-binding transcription factor in a phosphorylation dependent manner. The AHR/ARNT heterodimers bind to the aromatic hydrocarbon response element (AHRE) on the enhancer chromatin and the transactivation domains of the AHR facilitate promoter occupancy through the interaction with other components of the transcriptional machinery. Formation of an initiation complex at the promoter starts transcription of genes containing the AHRE such as *CYP1A1* and *1A2* (Whitlock, 1999).

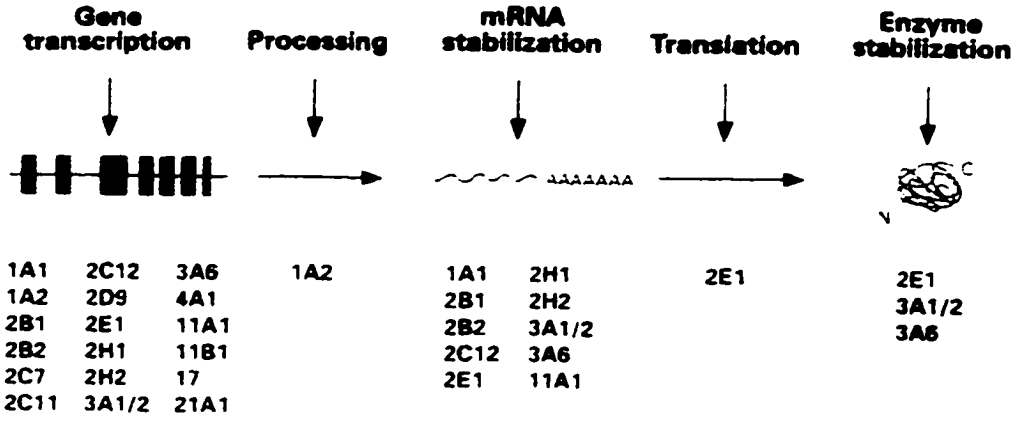
This results in the increased production or expression of these particular cytochrome P450 isoforms.

Various endogenous and exogenous compounds can modulate many of the other important cytochrome P450 isoforms. Transcriptional alteration is the most common mode of cytochrome P450 regulation, however, posttranscriptional modifications such as mRNA stabilization and protein degradation can also occur. Of all the isoforms, CYP2E1 has the most diverse mechanisms of regulation including transcriptional activation (during birth and fasting), mRNA stabilization (in diabetes), and protein stabilization (seen with chemical inducers) (Porter and Coon, 1991). Highlights of the important regulatory mechanisms for individual isoforms of the cytochrome P450 superfamily are illustrated in figure 1-1.

1.2.5 Expression of Cytochrome P450 in the CNS

The liver has classically been held as the major site of drug metabolism in the body. Evidence for the expression of multiple forms of cytochrome P450 in many extra-hepatic sites including the brain has led researchers to question the contribution of these enzymes to overall drug biotransformation. Taking into consideration the low level of cytochrome P450 in the brain (~0.5-2% of hepatic levels) and the limited access of substrates to the brain parenchyma, it is unlikely that these cytochrome P450 enzymes contribute significantly to the overall metabolism and excretion of drugs (Hedlund et al., 1998). The presence of these enzymes in the central nervous system leads one to speculate as to their function in physiological processes and pathological conditions. One of the first demonstrations of the brain's ability to support localized drug biotransformation was the

FIGURE 1-1



Diverse Modes of Cytochrome P450 Expression Regulation. This diagram illustrates the five main mechanisms by which cytochrome P450 expression can be regulated. For each mechanism, the isoforms susceptible to that particular type of regulation are listed below (taken from Porter and Coon, 1991).

observation that hypothalamic tissue could participate in the local production of neurosteroids (Fishman and Norton, 1975). Later, it was shown that the CNS was capable of *in situ* drug metabolism by demonstrating the N-demethylation of morphine in the hypothalamus, medial thalamus, and corpus striatum (Fishman et al., 1976). Coincident to these observations was the discovery of cytochrome P450 enzymes in the brain. Isoforms from families 1-4, considered to be the major drug-metabolizing enzymes and among the most abundant in liver, are expressed in brain tissue although they are only minor contributors to the overall content of cytochrome P450 in brain (Hedlund et al., 1998). Isoforms such as CYP11A1, involved in the synthesis of endogenous compounds, have been found in the brain and are thought to contribute significantly to the overall content of cytochrome P450 in the CNS (Hedlund et al., 1998). Unique isoforms of cytochrome P450 have also been described in brain tissue including CYP2D4, CYP7B and novel isoforms from the CYP3A and CYP4F subfamilies (Kawashima et al., 1995a; Kawashima et al., 1995b; Wang et al., 1996; Rose et al., 1997).

1.2.5.1 Subcellular Localization

Walther et al. (1986) described the subcellular distribution of cytochrome P450 isoforms in the brain. They found that cytochrome P450 enzymes were located both in the microsomal and the mitochondrial fractions obtained from brain tissue. Indeed it was determined that the majority of these enzymes reside in the mitochondrial membranes of cells and thus suggest an important role in the biotransformation of compounds such as fatty acids and steroids.

It is generally considered that cytochrome P450 enzymes that reside in the mitochondria are involved in biotransformation of endogenous compounds whereas isoforms that reside on the endoplasmic reticulum are specific for xenobiotic metabolism. Although this holds true in a global sense it is by no means strictly valid as many of the microsomal cytochrome P450 enzymes are involved, in varying degrees, in the modification of endogenous compounds (Lewis, 1996b). A similar trend is seen in the subcellular distribution of cytochrome P450 enzymes in the brain as alluded to above. CYP1A activity is typically considered to be associated with the microsomal fraction from cells. However, CYP1A activity has been measured in both microsomal and mitochondrial fractions from brain tissue indicating the overlap in function between the two cellular compartments (Walther et al., 1987a).

Although cytochrome P450 content is approximately five times higher in mitochondria than in the endoplasmic reticulum (microsomal fraction), it appears that the activity of brain cytochrome P450 towards exogenous compounds is higher in the microsomal fraction than in the mitochondria especially when activity is normalized to the content of cytochrome P450 (Perrin et al., 1990). Thus it appears as though a similar trend occurs in cellular distribution of cytochrome P450 enzymes in the brain as they do in the liver. The isoforms that reside on the endoplasmic reticulum are largely responsible for exogenous drug metabolism versus mitochondrial isoforms that are mainly involved in endogenous biotransformation.

1.2.5.2 Immunohistochemical Localization

The localization of cytochrome P450 enzymes in the brain has become the focus of much interest and research. However, many reports of distribution patterns are inconsistent in nature owing to the experimental limitations. Morse et al. (1998) indicate that immunohistochemical distribution often relates more to the type of antibody employed and thus makes the interpretation of the localization significance unclear. These problems necessitate the concomitant use of additional methods such as activity assays and/or *in situ* hybridization to confirm immunohistochemical data.

Despite these limitations, the localization of several cytochrome P450 isoforms has been described. The expression of the drug metabolizing enzymes, CYP1A1, 1A2, 2B1, 2E1, and 2D1 has been shown to occur in low levels in neuronal and glial cell populations throughout the brain parenchyma (Kapitulink et al., 1987; Kohler et al., 1988; Anandatheerthavarada et al., 1990; Hansson et al., 1990; Perrin et al., 1990; Schilter and Omiecinski, 1993; Norris et al., 1996; Riedl et al., 1996b; Morse et al., 1998).

CYP1A1 and 1A2 isoforms have been localized to many areas of the brain including the olfactory bulb, caudate nucleus, frontal cortex, thalamus, and hippocampus. These isoforms have also been found in neuronal cells derived from the substantia nigra, locus coeruleus, reticular formation, trigeminal nerve nucleus, and the dorsal motor nucleus of the vagus (Kohler et al., 1988). Interestingly, CYP1A1 and 1A2 have been shown to co-localize with GFAP (glial fibrillary acidic protein) positive glia indicating that astrocytes, as well as neurons, contain these isoforms of cytochrome P450. Much of

the CYP1A1/1A2 positive astrocytes were localized to areas within the forebrain and brainstem (Kohler et al., 1988).

Other studies failed to find the expression of CYP1A in the same pattern as described in the experiments by Kohler et al. (1987) (Kapitulink et al., 1987; Kohler et al., 1988; Morse et al., 1998). Immunohistochemical studies using different antibodies (raised against P450 purified from 3-MC treated rats or raised against the synthetic C-terminal peptide sequence of CYP1A1) found CYP1A expression in the globus pallidus, caudate putamen, amygdala, ventromedial nucleus of the hypothalamus, medial forebrain bundle, ansa lenticularis, and crus cerebri but clearly demonstrated that CYP1A expression did not arise from cell bodies of the substania nigra as found in the previous report (Kapitulink et al., 1987; Riedl et al., 1996b). Schilter and Omiecinski (1993) found high expression levels of CYP1A mRNA in the striatum and hypothalamus with lower levels present in the medulla oblongata, cortex and cerebellum. Later experiments by Morse et al. (1998) used activity and Western blotting to examine regional distribution of CYP1A1 and 1A2. They found activity and expression of these isoforms in microsomes from the cortex, cerebellum, brainstem, thalamus, hippocampus, and striatum. In addition, they also showed activity in the meninges (arachnoid and dura mater) and circumventricular organs (choroids plexus, pineal gland, median eminence, and pituitary) (Morse et al., 1998).

Localization of the cytochrome P450 isoform CYP2D1 (CYP2D6 in humans) in the brain has sparked considerable interest because of its putative role in the onset of Parkinson's disease (Tanner, 1989). In a comprehensive study by Norris et al. (1996), they reported a wide and constitutive distribution of CYP2D1 mRNA and protein in

neuronal and glial populations in the brain. However, this distribution was not confirmed by another study using antibodies raised against the C-terminal peptide of CYP2D1 and it was suggested that the earlier results may reflect cross-reactivity of the antibody with other isoforms from the CYP2D family (Riedl et al., 1996b).

Two other important drug metabolizing isoforms of cytochrome P450, CYP2B1 and CYP2E1 have also been immunohistochemically localized within the brain parenchyma. Much controversy has surrounded the localization of CYP2B1 in brain owing to the problem of non-specificity of the antibodies employed in the various studies (Hedlund et al., 1998). Using RT-PCR, CYP2B1 mRNA has been detected in the striatum, hypothalamus, and cerebellum, but levels are very low and do not reflect the substantial levels of CYP2B1 protein detected in previous studies (Schilter and Omiecinski, 1993). In keeping with these results for CYP2B1 mRNA, Riedl et al. (1996) used antibodies targeted against the CYP2B1 C-terminal peptide sequence to demonstrate highly localized expression in the neuronal cells bodies and projections in the paraventricular hypothalamic nucleus but not in other areas such as the basal ganglia (Riedl et al., 1996b). In contrast to results with CYP2B1, researchers have generally agreed upon the localization of CYP2E1 in dopaminergic neurons of the substantia nigra and in glial cells throughout the brain and in blood vessels (Hansson et al., 1990; Riedl et al., 1996b).

Owing to the variable results in cytochrome P450 localization in the brain, the true functional nature of these enzymes has been the subject of much speculation. Obviously, the localization of particular forms will govern the role it has not only in physiological functions but also its role in pathological conditions. Confirmation of

immunohistochemical results through other methods, such as *in situ* hybridization and activity assays performed on specific brain regions are necessary to elicit the true role of these enzymes.

1.2.5.3 Physiological Relevance

The previous section demonstrated that most isoforms of cytochrome P450 found in the brain tend to be localized in specific areas resulting in a distribution pattern that is no doubt physiologically significant. Mathematical modeling has shown that uniform distribution of the small amounts of cytochrome P450 in the brain would offer no protective advantage. However, increased localization of cytochrome P450 isoforms could lead to significant effects on xenobiotic concentration in specific areas (Britto and Wedlund, 1992). Examinations of putative functions of cytochrome P450 in the brain have indeed found this type of localization/functional relationship to be true.

Much of the evidence regarding the functional importance of cytochrome P450 in the brain focuses on its role in the synthesis of endogenous compounds. However, it has been shown that these enzymes also display characteristic drug metabolizing activities such as aryl hydrocarbon hydroxylase (AHH) activity (Walther et al., 1987a). This activity, while beneficial in some aspects, can be detrimental to the brain as CYP1A (AHH activity) can metabolically activate many environmental pollutants into reactive or carcinogenic products.

A similar phenomenon is seen with the expression and activity of CYP2D1. A susceptibility to Parkinson's disease has been correlated with a mutant allele in the rat *CYP2D1* gene, and thus the localization and activity of the associated protein in

dopaminergic tracts within the brain maybe critical to the understanding of this neurodegenerative disorder (Norris et al., 1996; Riedl et al., 1996b). It has also been shown that CYP2D isoforms are localized in a variety of areas within the brain including the thalamus, a site of action for opiates. Demonstration of the CNS to support the N-demethylation of morphine in opiate receptor rich areas such as the hypothalamus, medial thalamus, and corpus striatum led researchers to speculate that CYP2D isoforms may be responsible for this biotransformation of morphine and its subsequent effects on opiate receptor binding (Fishman et al., 1976; Mesnil et al., 1984; Norris et al., 1996). As well, a novel isoform from the CYP2D subfamily, CYP2D18 has been implicated in the local metabolism of imipramine, a tricyclic antidepressant (Kawashima et al., 1996). These observations have led to speculation of a role for these isoforms in the metabolism of many neuroactive agents (Ravindranath, 1998).

Other forms of the drug metabolizing cytochrome P450s such as CYP2B1 and CYP2E1 also have putative roles in the pathology of the brain. CYP2E1 activity is associated with several potential toxicological complications. These include the generation of significant amounts of free radicals that can be involved in lipid peroxidation and destruction of cellular membranes and, as well, this isoform is involved in the metabolism of a variety of organic solvents into potential neurotoxins (Warner and Gustafsson, 1994). In addition, the presence of CYP2E1 brings up significant concerns surrounding the formation of the toxin acetaldehyde from ethanol and the effects this compound may have on neuronal cells (Warner and Gustafsson, 1994). In a similar vein, the presence of CYP2B1 may be toxicologically important because of its capacity to

metabolize several important pro-carcinogenic compounds into active carcinogens and, as well, the metabolism of a variety of clinically relevant drugs (Hedlund et al., 1998).

Most of the cytochrome P450 content in the brain derives from isoforms that are involved in endogenous biotransformation (i.e. the mitochondrial isoforms). Many of these isoforms are involved in normal physiological functions. For example, the first step in steroidogenesis has been shown to occur inside the mitochondrial membranes of brain cells (Walther et al., 1987b). This rate-limiting step, the conversion of cholesterol to pregnenalone, is catalyzed by the cytochrome P450 isozyme, CYP11A1 and serves as the starting point for the synthesis of a variety of neuroactive steroids (Lewis, 1996b). CYP19 is another critical cytochrome P450 isoform involved in steroid synthesis. It mediates the conversion of androgens (adrostenedione and testosterone) to the aromatic estrogen compounds (estrone and estradiol respectively). The production of these estrogen compounds from androgens occurring in the diencephalons and limbic system may be essential for sex differentiation in neonatal rodents (Mesnil et al., 1984). These cytochrome P450 enzymes are not only involved in the synthesis of steroids but also in their inactivation through metabolic alterations. Thus these enzymes are capable of significant contributions to the duration of action of these types of endogenous compounds (Stromstedt et al., 1993). One such example of this type of inactivation occurs for the 3α-hydroxylation product of progesterone (3α-hydoxy-5α-pregnan-20-one (3α-OH-DHP)), a compound that exerts its potent anesthetic effects through the activation of GABA (γ-aminobutyric acid) receptors (Majewska et al., 1986). Cytochrome P450 isoforms (specifically, 3β-adial hydroxylase) metabolize 3α-OH-DHP into its inactive form thus terminating its physiological function (Stromstedt et al., 1993).

The other major role of mitochondrial cytochrome P450 isoforms is the ω-hydroxylation of fatty acids such as arachidonic acid (AA) and prostaglandins. AA is metabolized by isoforms of the CYP2C, 4A, and 4F subfamilies into a variety of different EETs, etc. (Hecker et al., 1995). These metabolites have been reported to participate in several important physiological functions by acting as intracellular signals involved in the release of somatostatin from the hypothalamus, aid in the regulation of cerebral blood vessel tone, and the stimulation of dopamine induced peptide hormone release (Warner et al., 1994; Kawashima and Strobel, 1995; Harder et al., 1997; Hedlund et al., 1998). Many of these endogenous functions are mediated by novel isoforms of cytochrome P450 whose expression in liver is very low or non-existent (Majewska et al., 1986; Stromstedt et al., 1993).

1.2.5.4 Inducibility

Discussed in an earlier section was the inducibility of cytochrome P450 in liver and in other extra-hepatic areas such as the lungs. Recently, evidence has been presented for the induction of various forms of cytochrome P450 in the brain from peripheral administration of chemical inducers such as β-naphthoflavone (βNF), phenobarbital, or ethanol. mRNA levels of CYP1A1/1A2 following treatment with βNF were substantially induced throughout the brain parenchyma (Schilter and Omiecinski, 1993). However, these results were not mirrored in activity levels or protein expression. Morse et al. (1998) did not find CYP1A1/1A2 activity and expression levels in brain parenchyma per se but did see substantially induced activity and expression in the circumventricular organs (areas with limited blood brain barrier due to their secretary properties) such as

the choroids plexus, pineal gland, pituitary, and median eminence. In addition, they found that treatment with βNF increased CYP1A1/1A2 activity and expression in areas adjacent to the blood-CSF barrier (arachnoid and dura mater) dramatically (Morse et al., 1998). The inability to significantly induce CYP1A activity in the brain parenchyma following treatment with βNF may be a protective function, as this could limit the formation of potentially reactive compounds in the CNS (Walther et al., 1987a).

Interesting induction patterns were also seen with phenobarbital inducible forms of cytochrome P450 (CYP2B1, 2B2, and 3A2). Examination of mRNA levels demonstrated an increase in CYP2B1 mRNA in the medulla oblongata, midbrain, and cortex and a concomitant decrease in mRNA for this isoform in the cerebellum, hypothalamus, and striatum. In comparison, CYP2B2 mRNA decreased in all regions while CYP3A2 mRNA was unchanged (Schilter and Omiecinski, 1993). CYP2B activity levels remained unchanged in the brain following treatment with phenobarbital mirroring the opposing effects on mRNA (Perrin et al., 1990). Miksys et al. (2000) showed an interesting example of a potentially important physiological function of cytochrome P450 by demonstrating that CYP2B1 could be induced in the brain following systemic administration of nicotine.

Although CYP2E1 is classically considered to be the ethanol inducible isoform of cytochrome P450, in the brain several isoforms of cytochrome P450 are induced in response to treatment with ethanol. Expression of isoforms CYP2C, 2D, 2E, and 4A were induced as a result of a single injection of ethanol (Warner and Gustafsson, 1994).

The induction pattern of cytochrome P450 isoforms in response to chemical inducers is significantly different than that seen in liver. The limited capacity to induce

isoforms of cytochrome P450 in the brain is no doubt a reflection of the specialized nature of this organ and its limited ability to regenerate and repair damage.

1.2.5.5 Expression in Humans

Investigation into the expression of cytochrome P450 isoforms in the human brain has just begun in earnest. To date several isoforms of cytochrome P450 have been detected in the brain parenchyma including both drug metabolizing and mitochondrial isoforms. Similar trends are seen in the expression of mitochondrial isoforms of cytochrome P450 in the human brain as in the brain of rodents. CYP26A, an isoform involved in the inactivation of all-*trans*-retinoic acid, has been localized in significant amounts in the cerebellum and pons of the adult brain (White et al., 2000). Other important isoforms involved in the synthesis of endogenous steroid hormones include CYP11A1, 17A, and 19. These isoforms are involved in the conversion of cholesterol to pregnenalone, pregnenalone to dehydroepiandrosterone, and the aromatization of testosterone and androgens to estrogens. Expression of CYP11A1 mRNA was detected in significant quantities in the hippocampus of patients with temporal lobe epilepsy and this expression was found to be sex-dependent with higher levels in women (Beyenburg et al., 1999). CYP17A activity has been found in a variety of cell types within the CNS including oligodendrocytes, astrocytes, and neurons (Brown et al., 2000). In a similar vein, mRNA levels of CYP19 were found in both the frontal and temporal neocortices and the hippocampus of the adult brain (Stoffel-Wagner et al., 1999). The presence and activity of these isoforms of cytochrome P450 in the human brain imply a role for them in the local production and/or metabolism of endogenous steroids.

Drug metabolizing isoforms of cytochrome P450 have also been detected in brain tissue. Currently, mRNA for isoforms from the CYP1, CYP2, and CYP3 families has been found in various areas of the normal adult brain (McFadyen et al., 1998). As seen in rats, cytochrome P450 content in the human brain is higher in the mitochondria than in the endoplasmic reticulum, possibly a reflection of its importance in the biotransformation of endogenous compounds. The metabolism of exogenous compounds however, was detected in the microsomal fractions indicating the preferential localization of these isoforms to the endoplasmic reticulum (Ghersi-Egea et al., 1993). Activity studies using alkoxyresorufins as substrates has shown the presence and functional activity of enzymes from the CYP1A, 2C and CYP3 families and subfamilies however, due to overlapping substrate specificity the particular isoforms present could not be determined (Ghersi-Egea et al., 1993). RT-PCR has led to the detection of specific isoforms of cytochrome P450 in different areas of the brain. A comprehensive study by McFadyen et al. (1998) looked at the expression of drug-metabolizing isoforms in various brain regions obtained during routine autopsies. The brain regions studied included frontal and temporal cortices, midbrain, cerebellum, pons, and medulla. CYP1A1 was detected in many areas of the brain examined while CYP1A2 was seen only in the basal ganglia (McFadyen et al., 1998). This type of distribution is particularly interesting as CYP1A2 in the liver is the constitutive form while 1A1 activity must be induced exogenously. CYP2C mRNA was found in a variety of brain regions and sequencing of the cDNA found the isoform to be CYP2C8 which is an arachidonic acid epoxygenase and may participate in a variety of physiological functions (Zeldin et al., 1995; McFadyen et al., 1998). The other isoforms they found in the human brain were

CYP2D6, 2E1, 3A4 and 3A5. The localization of these isoforms was more specific as CYP2E1 was only found in the medulla, while CYP3A4 and 3A5 were shown in the basal ganglia and frontal cortex (McFadyen et al., 1998). Of particular interest was the localization of CYP2D6 mRNA in the midbrain and reports that CYP2D6 can be found in neurons within the substantia nigra (Gilham et al., 1997; McFadyen et al., 1998). This has far reaching implications for the etiology of Parkinson's disease, which may be linked to a polymorphism in the *CYP2D6* gene.

1.3 The Immune System

The body has a complex, integrative system for protecting itself from invading microorganisms such as parasites, bacteria, and viruses. The immune system is comprised of two main arms, innate immunity, also called non-specific immunity, and adaptive or acquired immunity (commonly referred to as specific immunity). Innate immunity makes up the first line of defense against an invading pathogen and is composed of anatomic barriers (skin, mucus membranes), physiologic barriers (pH, temperature, chemical mediators), phagocytic/endocytic barriers, and inflammatory barriers (Kuby, 1997b). The adaptive immune response is further sub-classified into the humoral and cell-mediated immune responses. The humoral response refers to the activation of B-lymphocytes and the subsequent synthesis and release of specific antibodies into the serum (known in Latin as *humor*). The cell-mediated immune response activates T-lymphocytes that differentiate into T_H (helper) and T_C (cytotoxic) cells. Activation of the T_H cells is a critical step in adaptive immunity as they release chemical messengers (cytokines) that are necessary for activation of the T_C cells and the

differentiation of B cells in plasma cells. Both of these pathways only become active when stimulated by the presence of specific epitopes on an invading pathogen. B cells can be activated by the mere presence of the epitope whether it is on the bacteria itself or displayed by an antigen-presenting cell. In contrast, T cells only recognize epitopes from pathogens that are associated with MHC (major histocompatibility complex) molecules on the surface of antigen presenting cells (Kuby, 1997b).

To establish an infection, an invading pathogen must first overcome the physical and physiological barriers of innate immunity. The rapid response of the innate immune response comprises the phagocytosis of the pathogen and stimulation of the acute phase response. This process helps to restrict tissue damage to the site of infection or tissue injury and provides early protection of the host until the adaptive immune response can be stimulated. Activation of the adaptive immune response and the subsequent activation of T cells into T_C cells induce the differentiation of B cells into the antibody secreting plasma cells. The two arms of the immune system work together to eliminate the invading microorganisms and protect the host from further exposures (Kuby, 1997b).

1.3.1 Peripheral Acute Phase Response

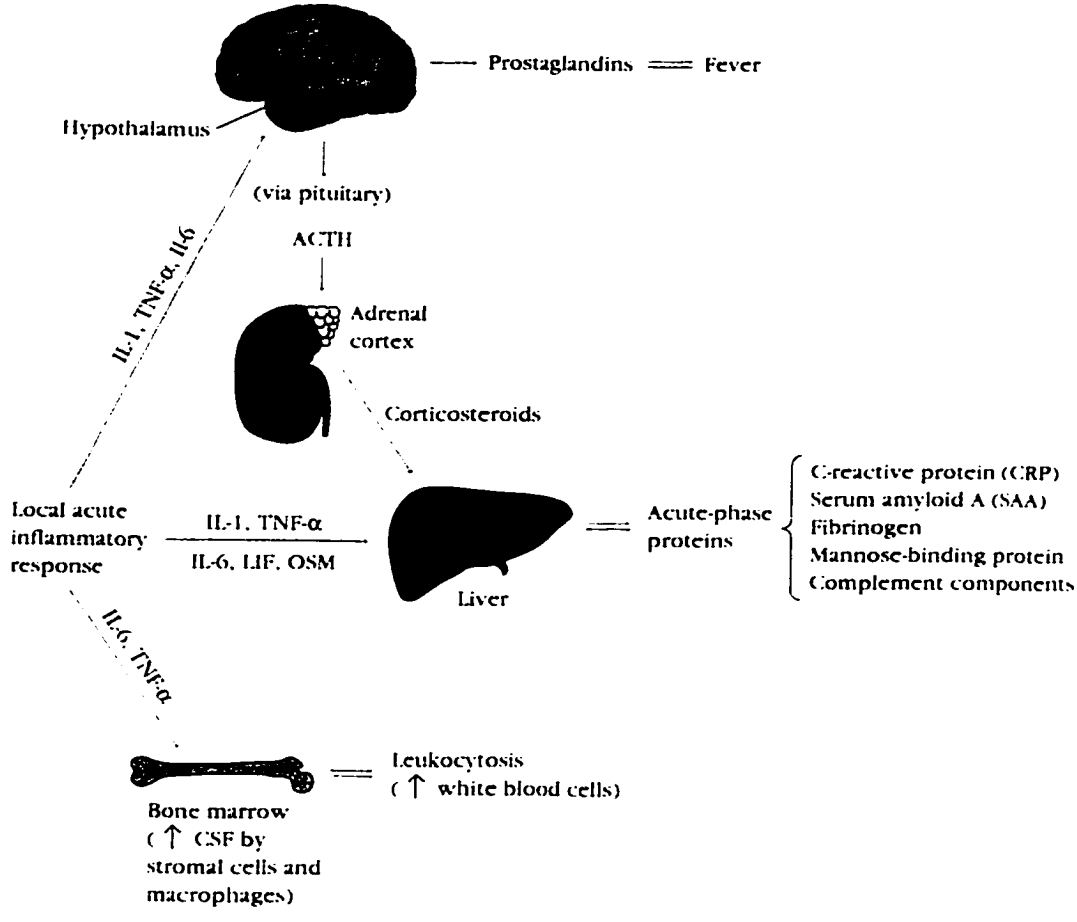
The local inflammatory reaction is accompanied by a systemic response known as the acute phase response. This is characterized by the induction of fever, increased synthesis of hormones, increased production of white blood cells, and the production of acute phase proteins in the liver (Kuby, 1997a). A localized inflammatory stimulus (either tissue damage or infection) induces vascular changes within the site of inflammation via the actions of mediators such as bradykinin, complement proteins, and

histamine, released from degranulated mast cells. These vascular changes cause neutrophils to adhere to endothelial cells within the site of injury where they can phagocytose the invading pathogens and release mediators that contribute to the inflammatory response (Kuby, 1997a). Amongst these mediators is macrophage inflammatory protein (MIP-1a and MIP-1b), a chemokine that attracts macrophages to the site of inflammation. Once active, macrophages participate in the phagocytosis of the pathogens and are capable of releasing the acute phase cytokines, TNF-α, IL-1, and IL-6. These cytokines exert both local and systemic effects in the inflammatory response. Their local effects include the facilitation and adherence of immune cells to the vascular endothelial cells through the induction of adhesion molecules such as E-selectin, ICAM-1, and VCAM-1, which promote adhesion of neutrophils and integrins on lymphocytes and monocytes (Merrill and Benveniste, 1996). The effects of these cytokines are highlighted in figure 1-2 and also include systemic effects such as stimulation of neurons within the hypothalamus to induce fever, the release of corticosteroids from the adrenal cortex, stimulation of acute phase protein synthesis, and actions on bone marrow to increase white blood cell production (Kuby, 1997a).

1.3.2 Acute Phase Response in the CNS

The brain has historically been considered an immunologically privileged area based on its physiological characteristics including the lack of a lymphatic system, very low expression of class I and II MHC molecules, and the presence of the blood brain barrier (BBB) (Merrill and Benveniste, 1996). In recent years it has become increasingly evident that the brain can mount a significant immune reaction in response to a variety of

FIGURE 1-2



Mediators and Effects of a Systemic Inflammatory Response. This diagram illustrates the diverse nature of effects the inflammatory response has on the host. Many of these effects arise from actions of inflammatory mediators on specific organ/cellular targets (taken from Kuby (1997a)).

stimuli. The inflammatory response in the central nervous system, however, is much subtler than its peripheral counterpart (Fig. 1-3). This is in large part due to the limited capacity for regeneration in the brain and the damage a robust immune response would cause. Tissue damage, or infection by bacterial or viral pathogens, in the CNS induces a characteristic inflammatory response within the brain parenchyma. Astrocytes and microglia become activated in response to immune challenges and undergo a process termed 'gliosis' (Andersson et al., 1992). This process is characterized by the increased expression of cell surface markers enabling these glial cells to become antigen presenting cells (Xiao and Link, 1998). In addition, they begin to synthesize and secrete a large variety of immune mediators including cytokines, chemokines, reactive oxygen species, nitric oxide (NO), and arachidonic acid metabolites (Minghetti and Levi, 1998). Prolonged activation of glial cells is considered a hallmark of many neurodegenerative diseases such as Alzheimer's and Multiple Sclerosis (MS) (Gonzalez-Scarano and Baltuch, 1999). In these types of diseases the inappropriate activation of immune cells within the brain can lead to the generation of substantial quantities of immune mediators such as cytokines, NO, and superoxide, all of which can have neurotoxic actions (Wood, 1998). It appears then, that an intricate balance is struck between the benefits of an inflammatory reaction in the protection of the brain from invading pathogens and the serious complications that arise from aberrant levels of immune activation. Given the deleterious effects that an inflammatory response can have in the brain, it is not surprising that this organ is generally resistant to induction of an immune response.

The ease and rapidity with which the peripheral system responds to an immune challenges by activation of the acute phase response contrasts greatly with the remarkable

FIGURE 1-3

Comparison of the Inflammatory Response in Systemic Tissues versus the Central Nervous System. **(A)** In the systemic tissues an inflammatory stimulus (1) can activate macrophages and mast cells or act directly on endothelial cells (EC). Stimulated mast cells release several preformed mediators such as histamine, leukotrienes, and platelet-activating factor while macrophages release cytokines (2). These mediators serve to activate the endothelial cells to increase the expression of adhesion molecules (3), increase the extravasation of plasma proteins (4), and promote the transmigration of neutrophils and monocytes. **(B)** In the CNS, an inflammatory stimulus activates microglia to release cytokines (1&2) that may act upon the brain endothelial cells (BEC). The exact effects these mediators have on the endothelial cells are under debate as neutrophil recruitment is absent and monocyte recruitment is generally delayed in CNS inflammation (taken from Perry et al., 1993).

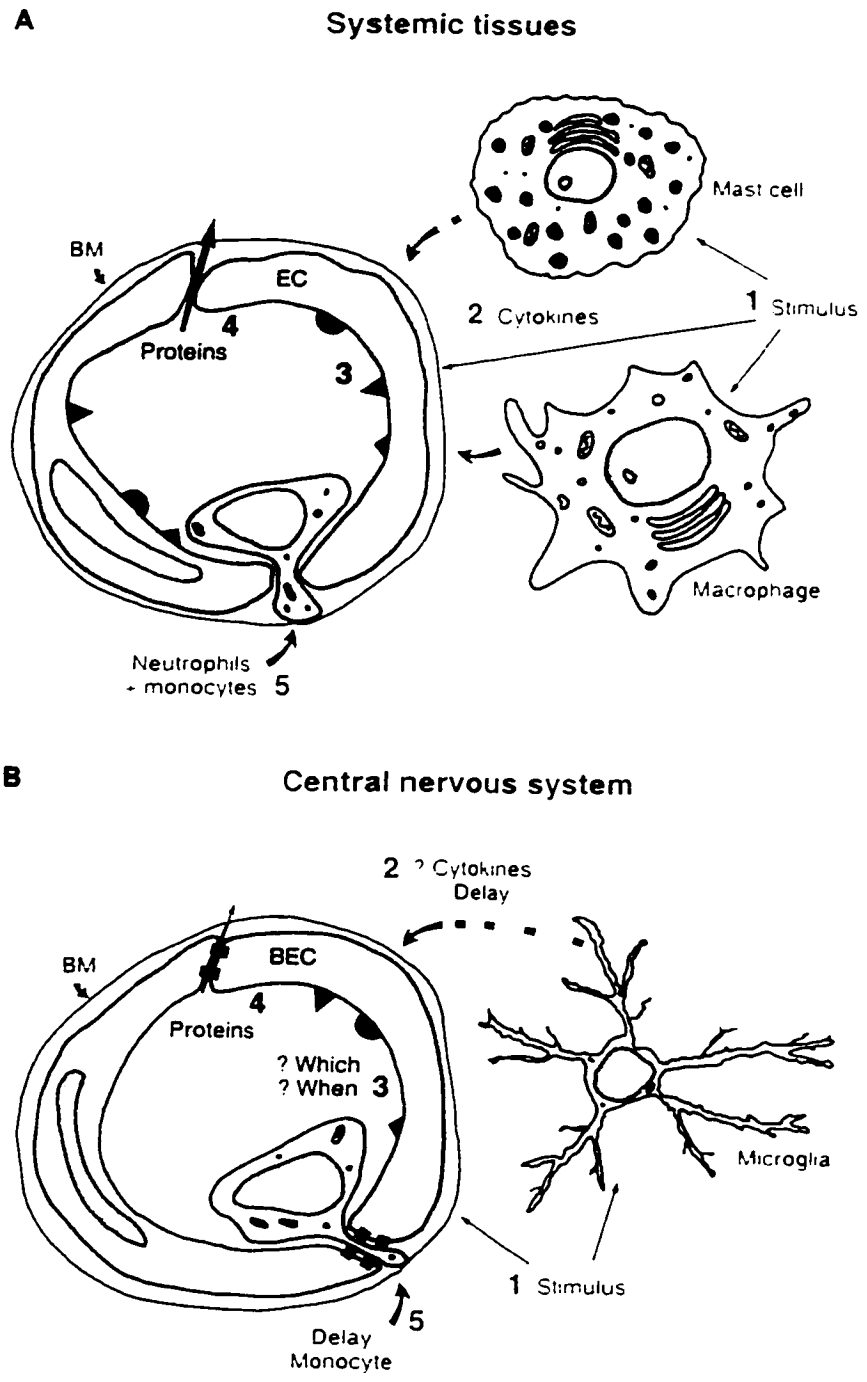

A
Systemic tissues
Mast cell
BM
EC
4
Proteins
3
2 Cytokines
1 Stimulus
Macrophage
Neutrophils
+ monocytes 5
B
Central nervous system
2 ? Cytokines
Delay
BM
BEC
4
Proteins
? Which
? When 3
Microglia
1 Stimulus
5
Delay
Monocyte

FIGURE 1-3

resistance of the brain against these actions. There are several possible explanations for this atypical response in the brain parenchyma including the down-regulated phenotype of microglia (resident macrophages of the brain) and the relative lack of infiltrating neutrophils and leukocytes (Perry et al., 1997). This lack of leukocyte infiltration into the brain parenchyma is the most striking difference between inflammation in the periphery and that of the CNS. Several hypotheses have been made to explain this phenomenon including the presence of the BBB, lack of adhesion molecule expression on the surface of cells, and/or the lack of chemokine expression. It was thought that neutrophils and monocytes were prevented from entering the CNS by the presence of an intact BBB. If the BBB were compromised and thus rendered more permeable this would facilitate the entry of immune cells. However, it has been shown that damage to the BBB is not sufficient for neutrophil recruitment into the CNS and thus this alone cannot explain the absence of these cells from the CNS (Perry et al., 1997). Evidence has been presented showing the upregulation of adhesion molecules (VCAM and ICAM) on the surface of cells in the BBB following a challenge with the bacterial endotoxin lipopolysaccharide (LPS). Despite the expression of these molecules, minimal leukocyte recruitment was seen suggesting that adhesion molecule expression is necessary but not sufficient for leukocyte entry (Perry et al., 1999). Recent evidence has shown that the expression of chemokines (chemoattractants for different populations of leukocytes) within the brain parenchyma may be involved in the lack of neutrophil recruitment. Alpha chemokines (C-x-C) are chemotactants for neutrophils and do not appear to be expressed in significant quantities in the brain (Perry et al., 1997). In contrast, beta chemokines (C-C) are released from astrocytes but are chemotactants for monocytes and lymphocytes that

have been observed in the brain during inflammation. Reports have shown high levels of chemokines expressed in the cerebrospinal fluid (CSF) of patients with chronic inflammatory diseases such as MS and that this corresponds to a high level of infiltrating leukocytes (Xiao and Link, 1998). This implicates leukocytes as having a role in severe and chronic inflammatory conditions but not in the acute inflammatory response in the brain.

In contrast to the lack of neutrophil recruitment, there does appear to be recruitment of peripheral macrophages into the brain. This appears to occur through an intact blood-brain barrier after a delay of about 48 hours (Perry et al., 1997). Upon entry into the CNS, these peripheral macrophages take on the down-regulated morphology of microglia characterized by low expression of cell surface antigens and reduced amounts of organelles involved in the synthesis and secretion of immune mediators (Perry et al., 1993).

Inactive T-cells are normally excluded from entry into the brain. In contrast, activated T-cells, regardless of antigenic specificity, can freely enter the CNS during normal physiological situations and in pathological conditions (Merrill and Benveniste, 1996). It is thought that these activated T-cells serve an "immunological-surveillance" function within the CNS during normal physiological conditions. If the activated T-cells recognize an antigen within the CNS, it will remain within the brain and participate in the inflammatory process through the release of pro-inflammatory cytokines TNF-α, IL-1, and in particular, IFN-γ, a cytokine that is involved in the upregulation of MHC class II molecules on perivascular macrophages, endothelial cells in the BBB, and pericytes (Fabry et al., 1994).

It is interesting to note that inflammatory reactions in various areas of the CNS can be extremely different. The brain parenchyma itself is remarkably resistant to the induction of an inflammatory response as described above. In contrast, inflammatory reactions in the meninges, ventricles and the choroids plexus are more characteristic of that seen in the periphery (Perry et al., 1999). For example, in response to an immune challenge in these areas there is a dramatic myelomonocytic recruitment within 12 hours (compared to 48 hour for the brain parenchyma). These differences are thought to arise from the differences in vasculature of these areas and the presence of macrophages that have an upregulated phenotype when compared to microglia. As well, the presence of mast cells in the meninges contributes to the differences seen in this area (Perry et al., 1993).

1.3.2.1 Astrocytes and Neuroinflammation

Glial cells constitute the largest proportion of cells within the brain parenchyma where they outnumber neurons approximately 8:1 (Chao et al., 1999). These glial cells are subdivided into macroglia (astrocytes and oligodendrocytes) and microglia with the vast majority of these glial cells (80%) being astrocytes. Astrocytes occur in two different forms denoted as type I (protoplasmic) and II (fibrous). Type I astrocytes are found in white matter where their "end feet" almost completely ensheath the brain capillaries which create tight junctions between cells and do not allow free passage of cells and small solutes into the brain (Aschner, 1998a). Type II astrocytes are found in both white and grey matter where they are intimately associated with neuronal surfaces around synapses and at the nodes of Ranvier (Barr and Kiernan, 1993; Tacconi, 1998).

These intimate interactions with cells of the CNS, suggest important roles for astrocytes apart from their structural support. Indeed, under normal physiological conditions astrocytes perform critical "house-keeping" functions. They function to aid in the removal of cell debris, serve to maintain normal potassium levels, release neurotropins, and help regulate glutamate neurotransmission and energy metabolism (Tacconi, 1998).

Astrocytes participate in the inflammatory response by undergoing reactive astrogliosis, a process that involves cellular hypertrophy and hyperplasia along with induction of glial fibrillary acidic protein (GFAP), the substance that constitutes filaments in glial cells (Ghirnikar et al., 1998). These reactive astrocytes are thought to mediate several beneficial functions in the inflammatory process including repair of the extracellular matrix, control of the BBB, and release of trophic factors for neurons (Ghirnikar et al., 1998). However, these beneficial effects are intricately balanced with detrimental effects such as the disruption of the normal function of astrocytes. This could result in compromised regulation of glutamate homeostasis in the extracellular fluid environment potentially contributing towards excitotoxicity (Lipton and Rosenberg, 1994). As well, their release of several cytokines and reactive compounds can be toxic to neuronal populations (Ghirnikar et al., 1998).

Reactive astrocytes are capable of releasing a substantial number of cytokines including IL-1α, IL-1β, TGF-α, TGF-β, IFN-α, IFN-β, IFN-γ, TNF-α, TNF-β, G-CSF (granulocyte colony stimulating factor), and GM-CSF (granulocyte-macrophage colony stimulating factor) (Ghirnikar et al., 1998). In addition to these cytokines, astrocytes are also the major source of the chemokines MCP-1 (monocyte chemoattractant protein) and

RANTES (regulated on activation, normal T cell expressed and secreted), which are responsible for leukocyte chemotaxis (Table 1-1) (Xiao and Link, 1998).

A role for astrocytes as antigen presenting cells within the brain has been gaining acceptance by researchers. MHC class I and ICAM-1 expression can be induced in astrocytes by the cytokines IL-1, TNF-α, and IFN-γ (Aschner, 1998b). This expression is thought to render them susceptible to lysis by cytotoxic T cells that may be a contributing factor to the development of autoimmune disorders (Aschner, 1998b).

1.3.2.2 Microglia and Neuroinflammation

In 1932, Pio Del Rio-Hortega first described microglia as a distinct cell type in the CNS based on its characteristic morphology (Gonzalez-Scarano and Baltuch, 1999). These glial cells constitute approximately 12% of all cells in the central nervous system and play an important role in the maintenance of homeostasis in the brain through their actions as the primary immune effecter cell of the CNS (Gonzalez-Scarano and Baltuch, 1999). Microglia are generally sub-divided into three types based on their location and morphology. Parenchymal microglia arise from differentiated monocytes that have entered the CNS during embryogenesis (Perry et al., 1999). This type of microglia is characterized by its down-regulated phenotype and its relatively low turnover rate. Perivascular microglia are typically found in the ventricles of the brain, and have a more upregulated phenotype compared to parenchymal microglia, a higher turnover rate, and are thought to be responsible for the initial contact with invading pathogens in the CNS (Gonzalez-Scarano and Baltuch, 1999). Leptomeningeal macrophages are the third type and exist within the meninges and are similar in characteristics to perivascular microglia.

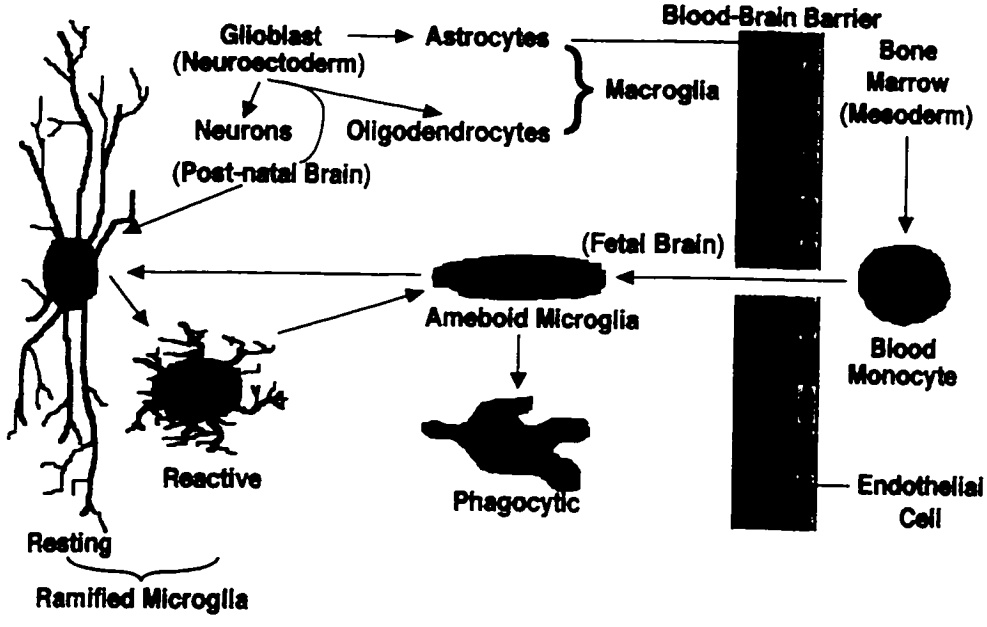
TABLE 1-1: Mediators Released by Glial Cells.
(Adapted from Xiao and Link, 1998).

Cytokines	Microglia	Astrocytes
IL-1β	+	+
IL-2	+	- or ?
IL-3	+	+
IL-6	+	+
IL-8	+	- or ?
IL-10	+	+
IFN-γ	+	+
TNF-α	+	+
TFG-β	+	+
LIF	+	- or ?
MIP	+	+
NGF	+	- or ?
PDGF	+	- or ?
M-CSF	+	+
GM-CSF	+	+
Surface Markers		
MHC	Inducible	Inducible
ICAM-1	Inducible	Inducible

Microglia have three different types of "activation states", ameboid, ramified, and reactive (Fig. 1-4). The ameboid form is an activated macrophage that has entered the brain during development and is considered to be the precursor to the ramified microglia (Peterson et al., 1997). Ramified, or resting microglia are usually found in the normal adult brain and are characterized by a small cell body with thin branching processes. In response to tissue disruption such as that seen during infection, inflammation, or traumatic injury, these ramified microglia can become transiently activated to phagocytose cell debris and release cytokines to protect the CNS from invading pathogens or tumour cells (Table 1-1) (Wood, 1998). These reactive microglia undergo significant morphological changes including a bushy or elongated appearance with a lack of processes and an upregulation in expression of cell activation markers (Peterson et al., 1997).

Microglia are normally in the ramified or resting state in the adult brain and become activated or reactive in response to serious pathological situations such as infection, inflammation, or tissue damage within the CNS (Peterson et al., 1997). Although this activity has intrinsic benefits in ridding the CNS from potentially harmful pathogens, it can also induce significant damage to nearby neurons. Indeed the hallmark of many neurodegenerative disorders is the presence of activated microglia. However, it is not known whether this increased activity is the cause of neurodegeneration or whether it results from the detection of neuronal damage induced by another mechanism (Perry et al., 1993; Gonzalez-Scarano and Baltuch, 1999).

FIGURE 1-4



Different Origins and Activation States of Microglia. Microglia are considered to arise from blood monocytes that enter into the brain during embryogenesis. In the mature brain these resting or ramified microglia are the normal morphological state. However, immune activation within the CNS can lead to the activation of resting microglia back into their ameboid then phagocytic form (from Peterson et al., 1997).

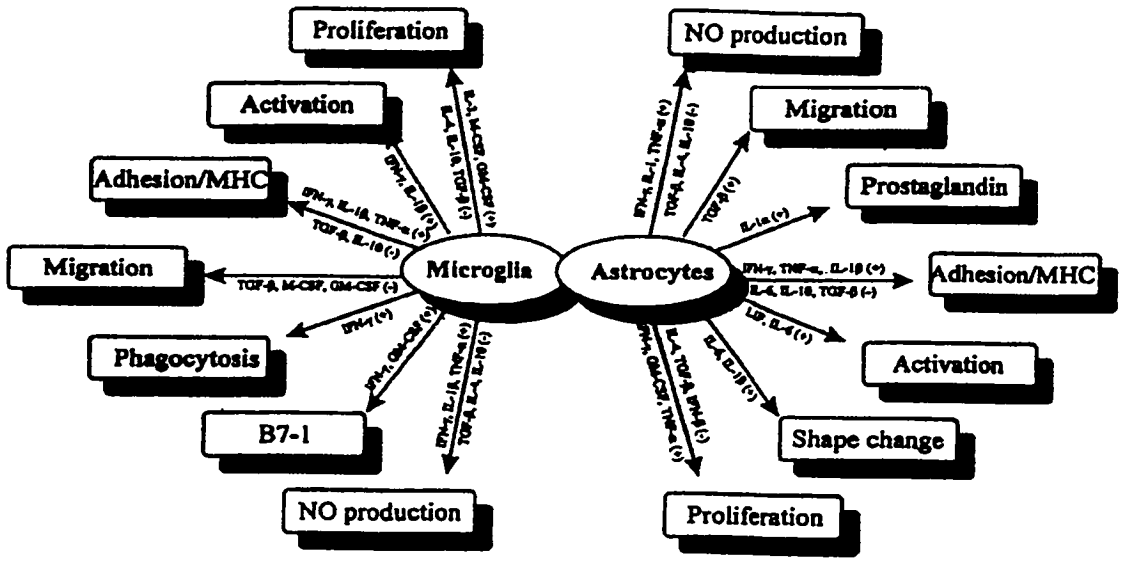
1.3.2.3 Cytokines and Neuroinflammation

Cytokines are the chemical messengers of the immune system. Through autocrine and paracrine interactions, they relay messages between cells of the immune system resulting in their coordinated activation and subsequent removal of the invading pathogen. The effects of cytokines on and within the peripheral nervous system have been well characterized during infection and inflammation (Kuby, 1997a). In contrast, the role of cytokines in the brain has just recently begun to be investigated in earnest. Current evidence has found that cytokines in the brain influence many processes such as behaviour, neuroendocrine activity, sleep, fever, psychic depression, and neurodegeneration (Xiao and Link, 1998). The participation of cytokines in these immune-mediated functions is not surprising, however what is not currently appreciated is whether cytokines have physiological roles in normal brain function. There is much controversy surrounding the answer to this question, as researchers have not been able to definitively demonstrate the presence of cytokines in the brain during homeostasis (Vitkovic et al., 2000). Some evidence does seem to demonstrate the presence of biologically active IL-1β in neurons and glial cells of specific areas in the normal brain. In contrast to IL-1β, the presence of TNF-α in the normal brain has not been convincingly demonstrated indicating that its levels are very low or absent (Vitkovic et al., 2000). The processing enzymes for IL-1β and TNF-α and receptors for these cytokines have been found in the brain demonstrating that a fully functional system for cytokine action does occur in the absence of an immune challenge. Currently there is evidence of a physiological role of cytokines in the normal brain as key regulators of NREM sleep (Vitkovic et al., 2000).

Of course the principal role of cytokines is their participation in the immune response. Following an immune challenge to the brain, cytokines are released from a variety of cells of which astrocytes and activated microglia are considered the major sources (Wood, 1998). However, endothelial cells lining the microvessels, pericytes, and neurons can also produce cytokines that may have important localized effects (Chung and Benveniste, 1990; Breder et al., 1993; Fabry et al., 1993). One of the earliest responses to an immune challenge is considered to be the activation of microglia and astrocytes and the subsequent release of immune mediators (Xiao and Link, 1998). Pro-inflammatory cytokines, released from these cells, act in an autocrine and paracrine manner to stimulate the cascade of cytokine release that is the basis of the inflammatory response in the CNS (Fig. 1-5) (Munoz-Fernandez and Fresno, 1998). The immune mediators released from activated microglia and astrocytes during the inflammatory process include but are not limited to proinflammatory cytokines (IL-1β, IL-6, TNF-α, and IFN-γ), anti-inflammatory cytokines (IL-10 and TGF-β), and growth factors (NGF and PDGF) (Table 1-1) (Xiao and Link, 1998).

Cytokines are notorious for both their redundancy and synergism in many of the effects they mediate. Cytokines are often seen to synergize with each other to induce the release of additional cytokines from immunocompetent cells. For example, IL-1β and TNF-α are both capable of releasing IL-6 from astrocytes; however, they have been shown to have a synergistic effect on IL-6 release when added together (Norris et al., 1994). In a similar manner, IFN-γ and IL-1β act in a synergistic manner to stimulate the release of TNF-α from astrocytes (Chung and Benveniste, 1990). These interactions between cytokine release and their effects create a positive feedback loop that augments

FIGURE 1-5



Responses of Microglia and Astrocytes to Cytokines. Cytokines released from activated microglia and astrocytes exhibit a myriad of effects that result in the immune response of the brain (adapted from Xiao and Link, 1998).

the immune response. These pro-inflammatory cytokines also induce the release anti-inflammatory mediators such as the growth factor TGF-β, which helps to limit the immune response and prevent excessive production of cytokines that would irreparably damage the CNS (Aloisi et al., 1999).

1.3.2.3.1 Interleukin-1

IL-1 is a collective term for two separate gene products, IL-1α and IL-1β, that share about 26% sequence homology (Rothwell, 1991). IL-1α and β tend to share the same biological activities primarily because they bind to the same receptor. However, evidence has been presented suggesting some differential actions of these two isoforms in mediating responses such as fever and hypothalamic-pituitary-adrenal (HPA) activation (Rothwell, 1991). This difference in activity may result from differences in their mode of expression; IL-1α is predominately membrane bound whereas IL-1β is the secreted form of IL-1 and probably accounts for the majority of effects (Martin et al., 1998). In response to infection or tissue damage, Il-1β is rapidly synthesized as pro-IL-1β then cleaved into its active form by the enzyme IL-1β converting enzyme (ICE) (Rothwell et al., 1999). IL-1α is also synthesized as pro-IL-1α but this form is biologically active and does not need any further processing. In addition to the two active isoforms of IL-1, there exists a third non-active form, IL-1ra (IL-1 receptor antagonist). This particular form binds to the type I IL-1 receptor (IL-1RI) with high affinity but does not transduce a signal and thus may be an important modulator of the effects of IL-1α and β (Martin et al., 1998).

Two receptors for IL-1 have been reported and are denoted as IL-1RI and IL-1RII respectively. IL-1RII is reportedly non-functional and thought to act as a decoy for biologically active IL-1α or β (Faggioni et al., 1995). However, it has been shown that type II IL-1 receptors are located in the hypothalamus and mediate the effects of IL-1 on fever (Rothwell et al., 1996). The other biological activities mediated by IL-1 forms are through the activation of the type I IL-1R. The signal transduction pathway for this receptor is highlighted in figure 1-6. Activation of this receptor by either IL-1α or β results it its association with accessory proteins and the eventual activation of TRAF-6 (TNF receptor associated factor). This results in several effects including the activation of nuclear transcription by stimulation of several factors including NF-κB (nuclear factor-κB), p42/p44, p38, and JNK (c-jun amino terminal kinase) (Rothwell and Luheshi, 2000). This IL-1 induced increase in transcription is most likely involved in the resulting increased synthesis of TNF-α and IL-6 (Chung and Benveniste, 1990; Norris et al., 1994). In addition, the sphingomyelin pathway has been implicated in IL-1β receptor signaling and in the actions of TNF-α. This pathway is initiated by the hydrolysis of the plasma membrane protein sphingomyelin to ceramide by the actions of the enzyme sphingomyelinase. Ceramide is thought to have a variety of intracellular actions including the stimulation of the MAP kinase pathway and the induction of NF-κB through the phosphorylation of I-κB (Kolesnick and Golde, 1994).

Microglia are reported to be the early primary source of IL-1 following tissue injury or infection whereas astrocytes produce IL-1 at a slightly later time point (Rothwell and Luheshi, 2000). This released IL-1 exerts a plethora of effects on the brain

FIGURE 1-6

Signal Transduction Cascade for IL-1 Proteins. Active forms of IL-1 stimulate IL-1 receptors (types I and II) of which the type I receptor has been better characterized. Binding of IL-1 to the type I receptor induces the association of several proteins resulting in the activation of MAPK (mitrogen-activated protein kinase) and NF-κB (via dissociation of the inhibitory I-κB). These processes result in the activation of several IL-1 responsive genes (taken from Rothwell and Luheshi, 2000).

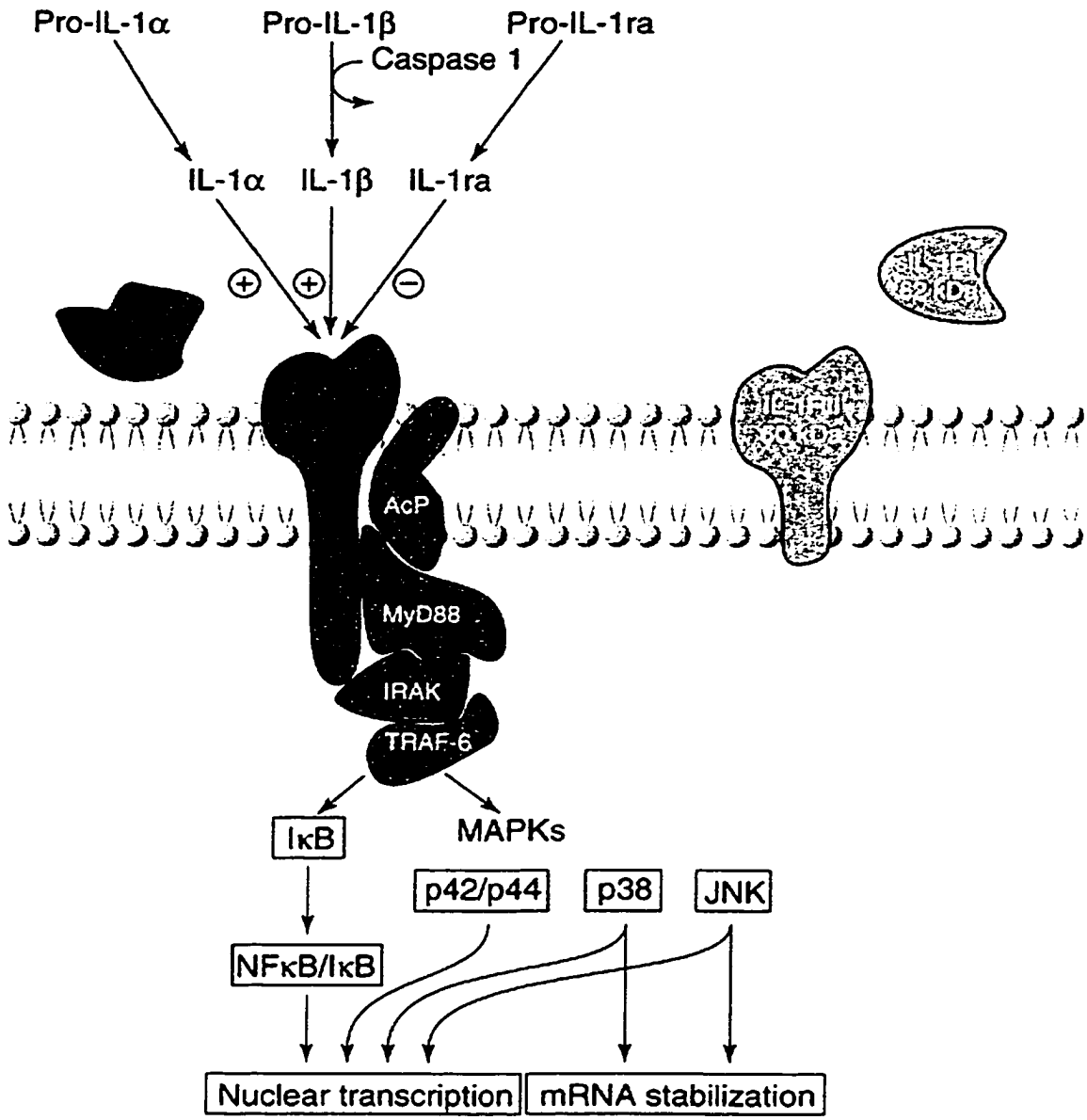

Pro-IL-1α
Pro-IL-1β
Pro-IL-1ra
Caspase 1
IL-1α
IL-1β
IL-1ra
AcP
MyD88
IRAK
TRAF-6
IκB
MAPKs
p42/p44
p38
JNK
NFκB/IκB
Nuclear transcription
mRNA stabilization

FIGURE 1-6

including but not limited to the induction of fever, hypophagia, slow-wave sleep, 'sickness behaviour', neuroendocrine changes (stimulation of the HPA axis through the release of CRH (corticotrophin releasing hormone) from the hypothalamus), stimulation of astrogliosis, and release of additional cytokines and immune mediators (Hofman, 1989; Aschner, 1998a; Rothwell and Luheshi, 2000). Resulting from the broad range of effects of IL-1 and the detection of this cytokine in the CSF and brains of patients with a variety of neurodegenerative disorders, researchers have begun to speculate that IL-1 may be intimately involved in the pathogenesis of these diseases (Hofman, 1989; Rothwell and Luheshi, 2000).

1.3.2.3.2 Tumor Necrosis Factor-α

TNF-α, a 17 kDa polypeptide, is another important pro-inflammatory cytokine released in the brain during an immune challenge (Aschner, 1998a). The source of this cytokine seems to depend on the type of injury incurred. For example, TNF-α is expressed in neurons after a penetrating injury or focal ischaemia, however following middle cerebral artery occlusion the main source is activated microglia (Rothwell et al., 1999). In response to infection or inflammation, TNF-α is secreted mainly from activated microglia and also reactive astrocytes and endothelial cells (Chung and Benveniste, 1990; Faggioni et al., 1995; Rothwell et al., 1996; Aschner, 1998a). TNF-α is synthesized in its membrane bound pro-TNF-α form until processed by the TNF-α converting enzyme into its soluble, mature form (Van Wagoner and Benveniste, 1999). TNF-α exerts a broad spectrum of effects on a variety of target cells resulting in both beneficial and detrimental effects. TNF-α increases the permeability of endothelial cells

lining the BBB and enhances their expression of adhesion molecules thereby promoting entry of immune cells from the periphery (Aschner, 1998a). As well, TNF-α induces MHC class I & II molecules and ICAM-1 on astrocytes facilitating their ability to become antigen presenting cells (Chung and Benveniste, 1990; Aschner, 1998a). One of the classic effects of TNF-α is the augmentation of the inflammatory response through the induction of the pro-inflammatory cytokine cascade. This occurs in the CNS by action of TNF-α on astrocytes and microglia to release IL-1, IL-6, G-CSF/GM-CSF, and itself (via autocrine actions) (Chung and Benveniste, 1990; Norris et al., 1994; Aschner, 1998a). TNF-α does seem to have some paradoxical actions in that it releases neurotrophic factors such as NGF from astrocytes and as well, potentially neurotoxic compounds such as glutamate, NO, and prostaglandins (Rothwell et al., 1996). TNF-α exerts these effects on the CNS through its interaction with its cell surface receptors. Two types of TNF-α receptors have been found in brain; TNFR1 (p55) and TNFRII (P75) (Beutler and van Huffel, 1994). TNFRII expression tends to be limited to myeloid, lymphoid, and endothelial cells whereas TNFRI is ubiquitously expressed and thus most signaling occurs through this receptor subtype (Paludan, 2000). To stimulate its receptor, TNF-α must form a trimer and interact with at least two TNFR molecules (Beutler and van Huffel, 1994). This induces the cytoplasmic domain (called the death domain) to interact with the adapter protein, TRADD (TNF receptor-associated death domain) which in turn associates with three other adapter proteins, TRAF-2, FADD (Fas-associated death domain), and RIP (receptor interacting protein). The downstream signals from FADD are involved in apoptosis whereas the TRADD/TRAF-2/RIP complex is responsible for NF-κB activation via phosphorylation and degradation of its inhibitory

protein IκB (Paludan, 2000). TNF-α also activates transcription factors AP-1 and IRF-1 (interferon regulatory factor) in some cells and may account for some of the synergy seen between TNFα and IFN-γ (Paludan, 2000). This signaling pathway is illustrated in figure 1-7 along with its interactions with IFN-γ.

As with many of the other cytokines, TNF-α is a "double-edged sword" as it has many beneficial effects and has also been implicated in the pathogenesis of neurodegenerative disorders such as MS through its toxic effects on oligodendrocytes (Chung and Benveniste, 1990). Therefore careful regulation of this cytokine is generally observed in the CNS.

1.3.2.3.3 Interferon-γ

Interferons occur as three separate classes produced by leukocytes (IFN-α), fibroblasts (IFN-β), and $CD4^+$ and 8^+ T-cells (IFN-γ) (Borgeson et al., 1989). IFN-γ is normally absent from the brain however, if an activated T-cell enters the brain and encounters its specific antigen then IFN-γ will be released (Fabry et al., 1994). As well, it can be released by astrocytes and microglia that are stimulated by TNF-α (Aschner, 1998a; Xiao and Link, 1998). IFN-γ has a variety of antiviral, anti-proliferative, and immunomodulatory effects (Aschner, 1998a). These immunomodulatory effects include the activation of macrophages, modulation of class I and II MHC expression, and differentiation of both T cells and B cells. Its upregulation of MHC class I and II molecules on astrocytes and microglia along with increased expression of ICAM-1 are necessary for their role as antigen presenting cells and thus in the activation of T cells

FIGURE 1-7

Signal Transduction Cascades for LPS, TNF-α, and IFN-γ. **(A)** Through activation of the Toll-like receptor (TLR), LPS stimulates the activation of the transcription factor NF-κB resulting in the transcriptional activation of LPS sensitive transcription sites. **(B)** IFN-γ simulates the tyrosine kinase activity of its receptor resulting in the phosphorylation of STAT proteins and their activation of the gamma-activating site (GAS). In addition, IFN-γ also stimulates the production of NF-κB. **(C)** TNF-α in a similar manner to IFN-γ and LPS, activates NF-κB and results in the transcriptional activation of TNF-α responsive sites on the gene (adapted from Paludan, 2000).

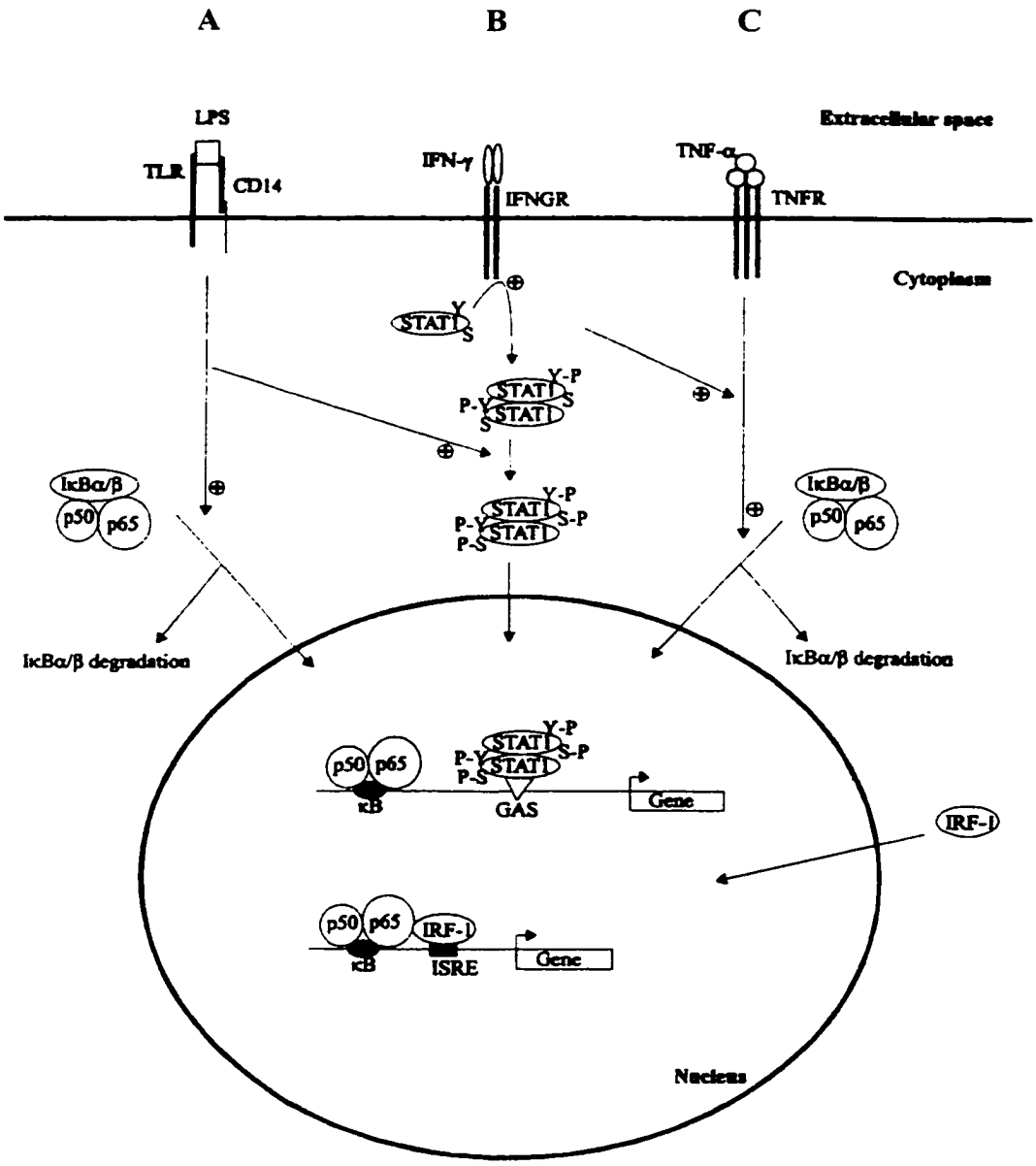

A
B
C
LPS
TLR
CD14
IFN-γ
IFNGR
TNF-α
TNFR
Extracellular space
Cytoplasm
STAT
Y-P
P-Y
S-P
P-S
IκBα/β
p50
p65
IκBα/β degradation
IκBα/β degradation
κB
GAS
Gene
IRF-1
ISRE
Nucleus

FIGURE 1-7

(Frei et al., 1987; Suzuki, 1997). IFN-γ induces the synthesis and release of reactive oxygen species including superoxide and NO from astrocytes and microglia and as well, inhibits COX-2 activation (cyclooxygenase-2) (Colton et al., 1992; Aschner, 1998a; Wood, 1998). IFN-γ is also known to provide a "priming signal" when astrocytes are exposed to suboptimal concentrations of immunogens such as endotoxin illustrating its synergistic potential and its pro-inflammatory function (Chung and Benveniste, 1990). As with the other cytokines, IFN-γ also induces the release of cytokines such as IL-1 and TNF-α from astrocytes (Chung and Benveniste, 1990; Aschner, 1998a).

The signal transduction pathway for IFN-γ is illustrated in figure 1-7. Stimulation of the IFN-γ receptor complex by ligand binding results in the activation of tyrosine kinases JAK1 and JAK2. These kinases phosphorylate tyrosine residues on STAT1 (signal transducer and activator of transcription-1) proteins that dissociate from the IFN-γ receptor complex. The STAT proteins form homodimers and translocate to the nucleus where they induce transcription via binding to the gamma-activating site (GAS) in IFN-γ inducible promoters (Paludan, 2000). Activation of the IFN-γ receptor also activates IRF-1 (interferon regulatory factor) that recognizes the sequence termed the interferon stimulation response element (ISRE) and plays a central role in IFN-γ induced gene expression (Paludan, 2000).

As discussed previously, IFN-γ synergizes with cytokines (especially TNF-α) and immunogens such as the endotoxin lipopolysaccharide (LPS). This synergy seems to arise from common and complementary signaling pathways as illustrated in figure 1-7 (Paludan, 2000). It is perhaps for these reasons that IFN-γ has such dramatic effects on

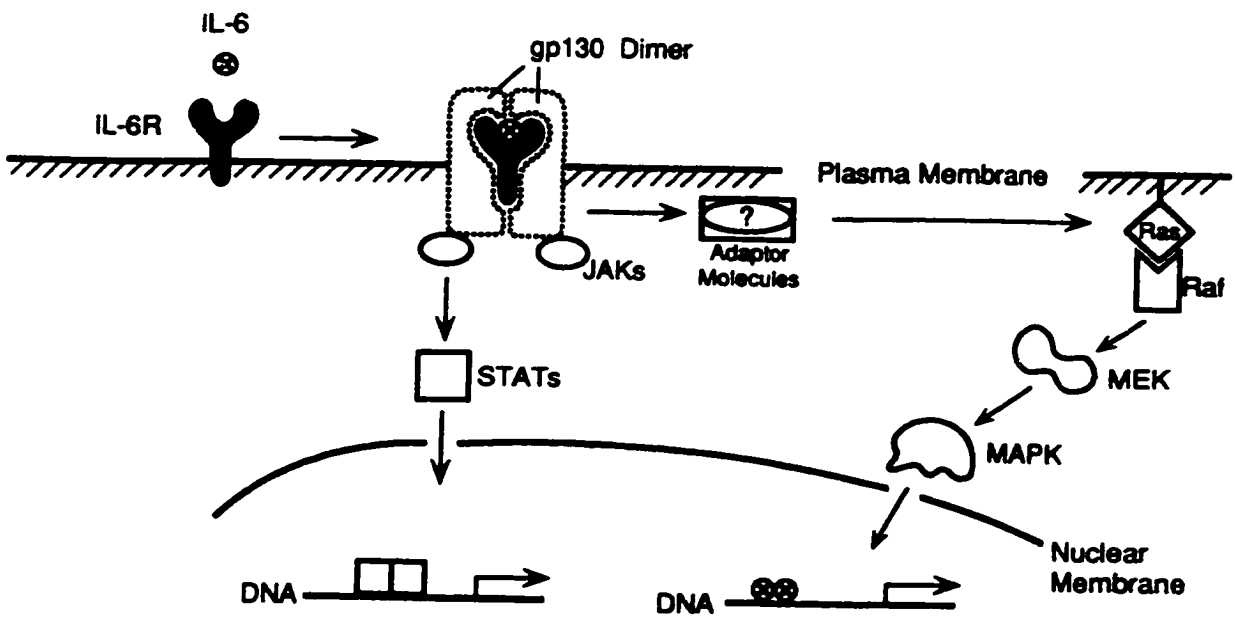
immune stimulation and is implicated in the chronic neuroinflammation characteristic of the neurodegenerative disorder MS (Frei et al., 1987; Aschner, 1998a).

1.3.2.3.4 Interleukin-6

IL-6 is a 26 kDa polypeptide that is synthesized and secreted from fibroblasts, monocytes, B cells, endothelial cells, and T cells (Aschner, 1998a). Several types of cells in the brain are capable of releasing IL-6 in response to different stimuli. Astrocytes can produce and secrete IL-6 in response to IL-1β, TNF-α, IFN-γ, and neurotransmitters (Benveniste et al., 1990; Norris and Benveniste, 1993). Microglia can also be stimulated to release IL-6 in response to bacterial and viral pathogens as well as cytokines (Norris and Benveniste, 1993). Interestingly, neurons have been shown to produce substantial amounts of IL-6 when stimulated by IL-1β and TNF-α or bacterial and viral pathogens (Benveniste et al., 1990; Ringheim et al., 1995).

IL-6 has been shown to have a variety of effects in the CNS during an immune challenge. This cytokine has been found to participate in astrogliosis via its mitogenic effect on astrocytes (Gadient and Otten, 1997; Aschner, 1998a). As well, IL-6 regulates several central effects such as fever, sleep, and food intake through its activation of the HPA axis (Gadient and Otten, 1997). In addition to the immune response related effects, IL-6 has been shown to aid in neuronal differentiation through its release of NGF from astrocytes (Aschner, 1998a). It is primarily secreted IL-6 that triggers another aspect of neuroinflammation, the production of acute phase proteins (class 1 & 2) in microglia and astrocytes (Wood, 1998).The signal transduction pathway for IL-6 is shown in figure 1-8. IL-6 acts on target cells through a receptor complex consisting of IL-6, gp130, and either

FIGURE 1-8



The Signal Transduction Cascade for IL-6. Activation of the IL-6 receptor by its ligand induces association with two molecules of gp130 and the activation of the JAK kinases. This results in the subsequent activation of two pathways, the stimulation of transcription by actions of the STAT proteins and activation of the MAPK pathway (taken from Gadient and Otten, 1997).

the IL-6R or the soluble IL-6R. The complex necessary for activation is a hexameric complex composed of two molecules each of ligand, receptor, and gp130 (Van Wagoner and Benveniste, 1999). This complex stimulates the activation of the tyrosine kinases, JAK1 and 2 of which JAK1 is the most important for IL-6 signaling. Activated JAKs then phosphorylate STAT-1α and STAT-3 proteins, which dimerize then translocate into the nucleus and activate IL-6 specific genes (Van Wagoner and Benveniste, 1999).
An additional pathway stimulated by IL-6 is the activation of the G-protein Ras that in turn will stimulate MAPK (mitogen-activated protein kinase) activity (Gadient and Otten, 1997). MAPK is often implicated in growth, and thus may mediate the effects of IL-6 on astrogliosis.

IL-6 has been reported to be intimately involved in several neurodegenerative disorders. High levels of IL-6 have been reported in the brains of patients with Alzheimer's or Parkinson's disease and also in patients with MS or HIV infections (Gadient and Otten, 1997). Interestingly, IL-6 has also been suggested to be involved in the growth and malignancy of tumours (Gadient and Otten, 1997).

1.3.2.4 Nitric Oxide and Neuroinflammation

Nitric oxide is a free radical gas that can easily diffuse through cell membranes to exert its effects. NO has a very short half-life and thus tends to act in an autocrine and paracrine fashion (Vanhoutte, 1987). NO is synthesized from L-arginine by the nitric oxide synthase (NOS) enzyme which occurs in separate forms that are coded by three distinct genes (Minghetti and Levi, 1998). Two of these forms of NOS are constitutively expressed and named for their cellular location, endothelial cells (eNOS) and neurons

(nNOS) (Kitamura et al., 1998). The third form is normally not expressed but can be induced in response to specific stimuli and thus has been termed inducible NOS (iNOS). The differences between these forms involve more than just their cellular location. Both eNOS and nNOS, the constitutive forms, require Ca^{2+} to stimulate their association with calmodulin resulting in enzymatic activation (Kitamura et al., 1998). Expression of iNOS is dependent on the *de novo* synthesis of its protein however, its activity is Ca^{2+} independent since calmodulin permanently and tightly binds to this enzyme at the low basal levels of intracellular Ca^{2+} (Minghetti and Levi, 1998).

The synthesis of NO occurs in an identical manner with all isoforms of NOS. The synthetic pathway occurs via NOS catalyzed transfer of electrons from NADPH (nicotinamide adenine dinucleotide phosphate) to the cofactors FAD (flavin adenine dinucleotide) then FMN (flavin mononucleotide) and eventually to the heme moiety on NOS resulting in molecular oxygen activation. Concurrent to this oxygen activation, the terminal guanidine nitrogen on L-arginine interacts with tetrahydrobiopterin forming a reactive intermediate that reacts with the activated oxygen to form NO, L-citrulline, and water (Minghetti and Levi, 1998).

One of the primary actions of released NO is its association with the heme moiety on guanylyl cyclase resulting in increased levels of the second messenger cGMP. This rise in intracellular cGMP can have a variety of effects including the relaxation of smooth muscle cells surrounding blood vessels (Lopez-Figueroa et al., 2000). Other effects of NO involve its interaction with reactive oxygen species and will be discussed later.

Although NO is synthesized in an identical manner with both constitutive and inducible forms of NOS, the amounts generated vary greatly. The constitutive forms,

nNOS and eNOS, produce low levels of NO in response to increased intracellular Ca^{2+} induced by cellular activation. This type of activation is particularly well suited for its role in the release of other neurotransmitters (nNOS) and its effects on blood vessel tone (eNOS) (Lopez-Figueroa et al., 2000). In contrast, the inducible form of NOS produces substantial and sustained levels of NO that are required for its role as a key mediator in the immune response (Liew, 1994; Lopez-Figueroa et al., 2000).

In the central nervous system iNOS is primary found in microglia and astrocytes (Boje and Arora, 1992). Interestingly, in rats the major source of NO is microglia with a lesser contribution by astrocytes whereas in humans, astrocytes are the main source with minor contributions by microglia (Minghetti and Levi, 1998). iNOS expression is induced in these cells by bacterial endotoxins (LPS) or cytokines such as IL-1β, IFN-γ, TNF-α and MIP (Galea et al., 1992; Murphy et al., 1993; Liew, 1994; Kitamura et al., 1998; Minghetti and Levi, 1998). Stimulation of immune responsive cells by these mediators induces iNOS expression rapidly (within 5 hours) and sustains this expression for approximately 24 hours. This results in detectable levels of NO around 6 hours and levels continue to increase linearly over the following 48 hours (Liew, 1994; Lopez-Figueroa et al., 2000).

Induction of iNOS transcription by immune mediators occurs through a variety of mechanisms. LPS activates iNOS expression through the stimulation of the membrane bound CD14 receptor resulting in activation of the second messenger systems such as MAPKs (ERK (extracellular signal-regulated kinase) & p38) and NF-κB (Xie et al., 1994; Galea et al., 1996; Bhat et al., 1998). In addition, cytokines can activate iNOS transcription through the activation of NK-κB, IRE, the TNF-α response element, NF-

IL6, and AP-1 sites contained on the iNOS gene (Kitamura et al., 1998; Minghetti and Levi, 1998). Since iNOS is involved in the immune response, it can be activated by several of these factors and thus transcriptional activation can occur through a variety of different and overlapping mechanisms.

The immunological capacity of NO was first demonstrated in 1985 by Stuehr and Marletta when they demonstrated that macrophages could be stimulated *in vitro* by LPS and IFN-γ to produce NO as measured by the appearance of nitrite and nitrate, the stable end-products of NO. They showed that this release of NO was involved in the inhibition of tumour cell replication (Stuehr and Marletta, 1985). This observation stimulated intense research into the role of NO in the immune response and has revealed its many effects, both beneficial and detrimental. The liberation of NO from cells activated by immune stimuli serves important anti-microbial and anti-neoplastic actions through its production of reactive nitrogen and oxygen species (Liew, 1994). However, production of these reactive species has also been shown to have cytostatic and/or cytotoxic actions on host cells through DNA strand breakage, inactivation of iron containing enzymes (may inhibit important enzymes in the mitochondrial respiratory chain), and depletion of ATP (Minghetti and Levi, 1998). These detrimental effects of NO have led researchers to postulate putative roles for NO in the pathogenesis of a variety of neurodegenerative disorders including MS, viral and immune-mediated insults where clinical symptoms have been shown to correlate with levels of iNOS mRNA (Koprowski et al., 1993; Minghetti and Levi, 1998). The balance between beneficial and detrimental effects seems to rely heavily on the amounts of NO produced in response to specific stimuli. Lower levels of NO release have been shown to function in a neuroprotective role in

cases of cerebral cortex damage due to ischaemia-reperfusion whereas the cytotoxic effects occur largely when high levels of NO are produced (Minghetti and Levi, 1998). This is primarily a result of its interaction with reactive oxygen species to produce the highly reactive compound peroxynitrite ($ONOO^-$). This molecule seems to target neucleophillic centers for nitrosylation. Thiol groups and transition metals contained in enzymes and proteins within cells are particularly susceptible to nitrosylation by peroxynitrite, a reaction that alters their normal biological function (Kitamura et al., 1998). Due to these potentially serious effects of NO on host cells, its regulation is normally very tightly controlled however, if disregulation occurs this can lead to some of the pathologies seen in chronic inflammation and neurodegenerative disorders (Liew, 1994).

1.3.3 Lipopolysaccharide

Escherichia coli lipopolysaccharide (LPS) is a bacterial endotoxin found on the outer membrane of gram-negative bacteria (Ryan, 1991). It is composed of four structurally and functionally distinct regions, the O-specific chain, the outer and inner cores, and the lipid A region (Sweet and Hume, 1996). The toxicological activity of this endotoxin is normally associated with the lipid A component of the LPS molecule as this region activates macrophages by binding to specific cell surface receptors. These receptors include CD18, the scavenger receptor (acetyl-low-density lipoprotein), and CD14. Both CD18 and the scavenger receptor are involved in the recognition, engulfment, and subsequent degradation of LPS, whereas the CD14 receptor has been implicated in the secretary responses of macrophages when exposed to LPS (Wright,

1991). CD14 receptors exist in two forms; in myeloid cells, CD14 is expressed as a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein. In contrast, a soluble form of CD14 lacking the GPI tail occurs in serum and is responsible for LPS activation of non-myeloid cells (Sweet and Hume, 1996).

1.3.3.1 Signal Transduction Pathways for LPS

Binding of LPS to the CD14 receptor is greatly facilitated when LPS is bound (opsinized) with LBP (LPS binding protein) (Wright et al., 1990). Low levels of LBP are normally found in serum and are induced during periods of immune activation (Kielian and Blecha, 1995). This LBP-LPS interaction is not an absolute requirement for activation of CD14 receptors, as higher concentrations of LPS can activate this receptor in the absence of LBP. High concentrations of LPS can also activate a CD14-independent signaling pathway suggesting that CD14 receptor activation is not the sole mechanism of LPS induced signaling in macrophages (Kielian and Blecha, 1995).

Investigation of the signal transduction pathway for LPS has been limited by several factors such as the complex nature of LPS and more specifically, the diverse types of responses seen in LPS activated cells from different species (Sweet and Hume, 1996). This diversity in cellular response to the same ligand has made the interpretation and comparison of results between studies very difficult. It has been shown that activation of protein tyrosine kinases (in particular the *src* tyrosine kinases, $p53/56^{lyn}$) are necessary for some of the effects of LPS acting through CD14 receptors (Sweet and Hume, 1996). These tyrosine kinases can activate ras and promote its interaction with Raf1 which in turn results in the phosphorylation of MAPK and stimulation of

downstream events such as the transcriptional activation of the TNF-α and IL-1β genes through the induction of transcription factors such as AP-1 and Elk (Reimann et al., 1994). However, this appears not to be the sole pathway stimulated by LPS as the activation of various transcription factors such as NF-κB and NF-IL-6 also occurs (Zhang et al., 1994). Recent evidence has shown that the LPS-LBP complex, acting through the CD14 receptor, stimulates the Toll-like receptor (TLR) 4 (Beutler, 2000; Paludan, 2000). This activation results in the docking of MyD88 and IRAKs (IL-1 receptor-associated kinases) to TLR followed by recruitment of TRAF6. TRAF6 then stimulates the downstream events such as activation of NIK (NF-κB-inducing kinase) resulting in the phosphorylation of the inhibitory protein I-κB and subsequent activation of NF-κB (Beutler, 2000). The putative signal transduction cascade for LPS is shown in figure 1-7 (note also the similarity to the IL-1β signaling cascade – Fig. 1-6) and reviewed in more detail in Beutler (2000). Much of the evidence surrounding the effects of LPS on macrophages is complex with many overlapping cascades. This serves to highlight the multitude of effects LPS exerts on immunocompetent cells. Although the delineation of the signal transduction cascade has been examined almost entirely in macrophages, similar mechanisms and pathways would most likely be seen in other cells such as microglia and astrocytes as the effects are generally thought to be mediated through CD14 receptors (Galea et al., 1996).

1.3.3.2 Mechanisms of LPS Induced Inflammation

LPS is considered a prototypical immune activating agent that has long been used in experimental animals to examine the effect of bacterial infection and inflammation

(Morgan, 2001). The inflammatory response induced by LPS in the periphery has been well characterized. One of the pivotal effects of LPS in the induction of an immune reaction is its activation of the pro-inflammatory cytokine cascade. This generally occurs in a time dependent manner with TNF-α release occurring first followed by IL-1β, IL-1α, and finally IL-6 (Chensue et al., 1991). These immune mediators stimulate a diverse number of effects as discussed in section 1.3.1. The actions of LPS on the central nervous system are similar in principle to those in the peripheral system with the notable exclusion of neutrophils and a delay in macrophage recruitment (Perry et al., 1993). Many of the aspects of central inflammation, including those stimulated by LPS, have been discussed earlier and therefore these effects will only be briefly dealt with here. In a similar manner to the periphery, centrally administered LPS induces cytokine release within the brain in the same time-dependent manner (Stern et al., 2000). As well, LPS stimulates the production of other immune mediators such as NO and prostaglandins through the induction of enzymatic activity (Minghetti and Levi, 1998). Of particular interest is the ability of centrally administered LPS to exert substantial effects on the periphery. For example, i.c.v. injection of LPS induces the production of IL-6 in peripheral blood through an α-adrenergic receptor mechanism (Gottschall et al., 1992; Finck et al., 1997). In a similar manner, LPS can effect alterations in the HPA axis via stimulation of CRH from the hypothalamus and the induction of ACTH (adrenal corticotrophic hormone) from the pituitary. Substantial levels of ACTH have been measured in peripheral plasma in response to centrally administered LPS demonstrating that inflammatory conditions can and do affect both the central and peripheral systems regardless of the point of origin (Habu et al., 1998).

1.4 Effects of Inflammation on Cytochrome P450

1.4.1 Historical Perspective

The interaction of the immune system with the drug metabolizing system was first observed by Samaras and Dietz (1953) who observed that pentobarbital induced sleep times were augmented in rats that were pretreated with trypan blue dye, an activator of the reticulo-endothelial system. Approximately 20 years later, Morahan et al. (1972) demonstrated that the interferon inducer, polyriboinosinic-polyribocytidylic acid (Poly (I,C)) dramatically depressed levels of microsomal drug metabolism. Subsequently Chang et al. (1978) reported one of the first recognized clinical interactions between drug metabolism and the immune response. They showed a correlation between the incidence of upper-respiratory-tract infection and the altered pharmacokinetics of theophylline in asthmatic children. Kraemer et al. (1982) confirmed this finding during an outbreak of influenza B where they found an increased prevalence of toxic levels of theophylline in virally infected asthmatic children. Subsequent experiments determined that it was not the virus itself that altered theophylline metabolism, rather it was interferons, a group of antiviral cytokines released in response to viral infections that mediated these effects (Renton, 1981b; Singh et al., 1982). Since these early reports, similar interactions between these two systems have been described for infections induced by bacterial, viral, or parasitic pathogens, inflammatory agents, and purified agents such as LPS (reviewed in Iber et al. (1999).

1.4.2 LPS and Cytochrome P450

Effects of immune activation on cytochrome P450 expression and activity have been explored using a variety of models of which the most popular involve the administration of LPS (Morgan, 2001). When injected systemically, LPS has been shown to alter the expression and/or activity of a variety of cytochrome P450 isoforms (Stanley et al., 1988; Morgan, 1989; Barclay et al., 1999). LPS has differential effects on cytochrome P450 expression and activity depending on the isoform examined. In the majority of cases, cytochrome P450 activity is suppressed in response to LPS, however there are notable exceptions. Isoforms from the CYP4A subfamily, which regulate fatty acid hydroxylation, are generally induced in response to LPS (Sewer et al., 1996). Another interesting exception is that of the CYP2E1 isoform. Its regulation by LPS seems to depend more on the location of the enzyme as hepatic CYP2E1 is suppressed in response to LPS whereas, CYP2E1 in astrocytes and its mRNA in kidneys are induced in response to exogenously administered LPS (Sewer et al., 1996; Tindberg et al., 1996). This raises the interesting possibility that these differential effects on cytochrome P450 by infection or inflammation occur for specific physiological functions as opposed to a non-specific consequence of immune activation. The functional significance of altering cytochrome P450 activity during the acute phase response has been raised and several possible reasons for this type of regulation have been suggested (reviewed in Morgan (2001)). One of the potential explanations is that cells must dedicate their transcriptional machinery to the synthesis of acute phase proteins (Morgan, 1989). Other explanations revolve around the generation of reactive oxygen and nitrogen compounds, specifically, the generation of superoxide and NO from cytochrome P450 catalyzed reactions and

through the induction of iNOS by LPS. These reactive species can form peroxynitrite, a highly reactive compound known to nitrosylate proteins within the cell (Bolanos et al., 1997). The potentially serious disruption of normal cell function that can occur as a result may be another reason why cytochrome P450 isoforms are downregulated in response to infection or inflammation. Although these observations give viable explanations as to why isoforms of cytochrome P450 are downregulated during the acute phase response, they do not account for the differential effects seen with the various isoforms and thus the reasons for the alterations in the cytochrome P450 system by infection and inflammation still remain under investigation (Morgan, 2001).

As discussed earlier, LPS is frequently used as a model of inflammation and thus reproduces many of the effects seen during this condition such as cytokine release (Chensue et al., 1991), induction of iNOS (Garthwaite and Boulton, 1995), increased COX-2 activity (Minghetti and Levi, 1998), stimulation of acute phase protein synthesis (Morgan, 1989), and activation of the HPA axis and subsequent release of glucocoticoids (Habu et al., 1998) amongst others. Early experiments with LPS sought to reveal whether the effects on cytochrome P450 were due to an immune mediator released by LPS or whether these effects were due to LPS *per se*. Using an LPS resistant strain of mouse, Ghezzi et al. (1986c) and later Shedlofsky et al. (1987), showed definitive proof that the effects of LPS were mediated through the release of intermediate products rather than through a direct action of the endotoxin. Exogenous administration of IL-1 emulated the effects of LPS in the LPS-resistant mouse strain implicating cytokines and specifically IL-1 as one of the mediators of LPS' effects on cytochrome P450. These experiments were some of the earliest demonstrations that the regulation of cytochrome

P450 by infection and inflammation is mediated through the stimulation of intermediate products such as cytokines. Subsequent research has demonstrated that other immune mediators such as NO, prostanoid products, and glucocorticoids also play a role in this response (Moreno et al., 1991; Iber et al., 1997; Ferrari et al., 2001).

1.4.3 Cytokines and Cytochrome P450

1.4.3.1 Evidence for Immune Mediators

Ghezzi et al. (1986c) demonstrated that conditioned medium from human monocytes stimulated with LPS could depress CYP1A activity in cultured hepatocytes. In a similar manner, Peterson and Renton showed that Kupffer cells (the resident macrophages of the liver) released a soluble factor in response to dextran sulphate or latex particles that was capable of depressing cytochrome P450. Indeed they found that Kupffer cells were a requirement for this effect, as dextran sulphate or latex particles had no effect on hepatocytes alone (Peterson and Renton, 1984; Peterson and Renton, 1986). Barker et al (1992) showed similar effects on induced levels of CYP1A1 and 1A2 mRNA in primary cultures of rat hepatocytes treated with conditioned medium from phorbol ester-activated human peripheral blood monocytes. This effect was mimicked by adding IL-1β lending further credence to the hypothesis that cytokines mediate many of the effects of immunostimulants on cytochrome P450 (Barker et al., 1992). Effects of LPS on CYP1A activity in the murine hepatoma cell line Hepa1, were also shown to require the presence of macrophages. LPS did not have any effect on CYP1A activity in these hepatoma cells in the absence of monocyte-conditioned medium whereas exogenously administered TNF-α depressed CYP1A activity when added directly to these cells (Paton

and Renton, 1998). Clearly the effects of immune stimulants have been convincingly attributed to the activation of macrophages and the subsequent release of immune mediators such as cytokines.

1.4.3.2 *In Vitro* Effects of Cytokines

The advent of recombinant cytokines was a pivotal point in this area of research as the availability of these cytokines allowed investigators to determine the direct effects of cytokines on cytochrome P450 enzymes. Investigation of cytokine effects generally focuses on the roles of TNF-α, IL-1, and IL-6 as they are considered to be the major pro-inflammatory cytokines (Chensue et al., 1991). Many of these effects have been studied in cell culture systems as this provides a uniquely controlled environment in which to examine the effects of a single agent on a specific target. Cell lines in particular give a uniform cellular population and thus eliminate much of the inter-individual effects seen with primary cultures and *in vivo* models. In looking at the interaction between the immune system and cytochrome P450 enzymes, human hepatoma cell lines have been employed. Addition of recombinant human IL-6 has been shown to depress levels of CYP1A1, 1A2, and 3A3 mRNA and CYP1A1 protein in these cell lines (Fukuda et al., 1992; Fukuda and Sassa, 1994). Despite these observations and the ease of using cell lines, their limited expression of cytochrome P450 isoforms has led to the increasing popularity of primary cultures of hepatocytes as a model for examining the effects of cytokines on cytochrome P450 isoforms.

Isolated hepatocytes have been used as a model system in which to examine the effects of cytokines on cytochrome P450. Using cultured rat hepatocytes, Williams et al. (1991) showed that human IL-6 depressed phenobarbital induced CYP2B1/2 activity, mRNA, and immunoreactivity. Ferrari et al. (1992) demonstrated that IL-1β decreased CYP1A1 activity in primary cultures of rat hepatocytes. Interestingly, in their model, IL-1β did not depress constitutive CYP3A activity in rat hepatocytes but prevented its induction by dexamethasone (DEX) (Ferrari et al., 1992). Clark et al. (1995) used EROD and BROD assays as measures of CYP1A and CYP2B activity in cultured rat hepatocytes treated with pro-inflammatory cytokines. IL-1β and IL-6 were equipotent in decreasing phenobarbital induced CYP2B1/2 activity whereas IL-1α was less active. In a similar manner as Ferrari et al. (1992), they found that these cytokines decreased 3-MC induced CYP1A1/2 activity although these effects were less potent than those seen with CYP2B1/2. In a similar vein, IFN-γ decreased CYP2B1/2 activity whereas it had no effect of CYP1A1/2 activity (Clark et al., 1995).

Some striking similarities and stark contrasts have been seen with regards to the sensitivity of rat and human hepatocytes to cytokines. Abdel-Razzak et al. (1993) showed that IL-1β, IL-6, and TNF-α are the most potent depressors of P450 isoforms (CYP1A2, 2C, 2E1, 3A) in human hepatocytes. IFN-γ on the other hand depressed 1A2 and 2E1 mRNA and EROD activity only, indicating that cytokines have differential effects on the cytochrome P450 isoforms. This trend in differential regulation was seen on the 3-MC induction of CYP1A mRNA as IL-1β, TNF-α, IFN-γ, and IFN-α blocked the induction whereas IL-6 had no effect (Abdel-Razzak et al., 1994). Similar results were obtained by Mutané-Relat (1995) as they showed that TNF-α is a potent suppressor

of induced levels of CYP1A1 and 1A2 mRNA and protein in primary human hepatocytes (vs. IL-6 and IL-1α) whereas IL-6 was the most potent at suppressing rifampicin induced CYP3A4 mRNA and protein. IL-1α, on the other hand, had minor effects on either family (Muntané-Relat et al., 1995).

The differential effects of cytokines on cytochrome P450 activity reported in different studies result from several factors including some species differences, induced vs. constitutive expression of isoforms, different concentrations of cytokines, and most importantly the experimental time frame. A review by Morgan (1997) describes two different phases of cytokine effects; the primary phase is a result of the direct action of cytokines and occurs from 4-24 hours, whereas the secondary phase results from indirect action of cytokines and occurs at 48 hours and on. As a result, experimental observations must be carefully interpreted.

1.4.3.3 *In Vivo* Effects of Cytokines

Parkinson et al. (1982) and Singh et al. (1982) were among the first to demonstrate that recombinant human IFN-α could depress levels of cytochrome P450 in mouse liver. Subsequently, Franklin and Finkle (1985) showed that another subtype of interferon (IFN-γ) had effects on cytochrome P450 similar to human IFN-α. Interestingly, these same researchers found a species specificity with IFN-γ as murine IFN-γ depressed cytochrome P450 but human IFN-γ did not (Franklin and Finkle, 1986). Stanley et al. (1991) demonstrated that IFN-α had differential effects on constitutive and inducible isoforms of cytochrome P450. They showed that IFN-α potentiated the induction of CYP1A by 3-MC while it prevented induction of CYP2B and 2C by

phenobarbital demonstrating that regulation of cytochrome P450 enzymes by interferons seems to be isozyme specific. Subsequently it was shown that IFNs were not the only type of cytokine that could interact with the drug metabolizing system. Ghezzi et al. (1986) demonstrated that cytochrome P450, specifically the CYP1A isoforms, were depressed in response to treatment with recombinant IL-1 or TNF-α (Ghezzi et al., 1986b; Ghezzi et al., 1986c). Later experiments by this group demonstrated that DEX, a potent inhibitor of IL-1, prevented the decrease in CYP1A by LPS or TNF-α but did not have any effect on the IL-1 induced decrease in CYP1A activity. This led the authors to suggest that IL-1 was the critical mediator released from monocytes stimulated by either LPS or TNF-α (Bertini et al., 1989). Wright and Morgan (1991) demonstrated that low doses of DEX potentiate the effects of IL-1α on the suppression of hepatic female specific CYP2C12 expression. In subsequent years Morgan (1994) demonstrated that IL-1 in combination with low dose DEX decreased CYP3A2, 2C11 and 2E1 mRNA and protein.. Clearly then, modes of cytochrome P450 regulation by immune mediators such as cytokines and glucocorticoids are complex in nature. In the absence of DEX, IL-1β has been shown to decrease the activity and protein of CYP3A1 (Ferrari et al., 1993), CYP1A1, 2B1/2, 3A, and 1A2 in arthritic rats (Ferrari et al., 1993), and 2D6 mRNA (Trautwein et al., 1992). The biologically related compound, IL-1α has been shown to depress CYP2B1, male specific-2C11, 1A1, and the activity, mRNA, and protein of CYP2D (Kurokohchi et al., 1992). The biological relevance of results with IL-1α is complex as IL-1α is normally found intracellularly or attached to the cell membrane. Due to this restriction, the effects of IL-1α produced *in vivo* on cytochrome P450 activity are likely to be minor compared to IL-1β which is secreted by cells (Martin et al., 1998).

TNF-α has differential effects on cytochrome P450 including the down-regulation of CYP3A2 and 2C11 in male rat liver with no effect of CYP2A1 and 2C6 (Nadin et al., 1995). As well, TNF-α has been shown to depress CYP2D6 mRNA in male LPS resistant mice (Trautwein et al., 1992). In 1990, Poüs et al. demonstrated that IL-1β or TNF-α alone was sufficient to decrease total hepatic cytochrome P450 content however, when combined together their effect was more potent than either alone.

Of all the major acute phase cytokines, IL-6 seems to have the most variable effects on cytochrome P450 isoforms. IL-6 has been reported to depress CYP2D6 mRNA (Trautwein et al., 1992), CYP2C11, and 2E1 mRNA (Morgan et al., 1994). In contrast, IL-6 has been shown to have no effect on 3A1/2 and CYP2B activity (Chen et al., 1992).

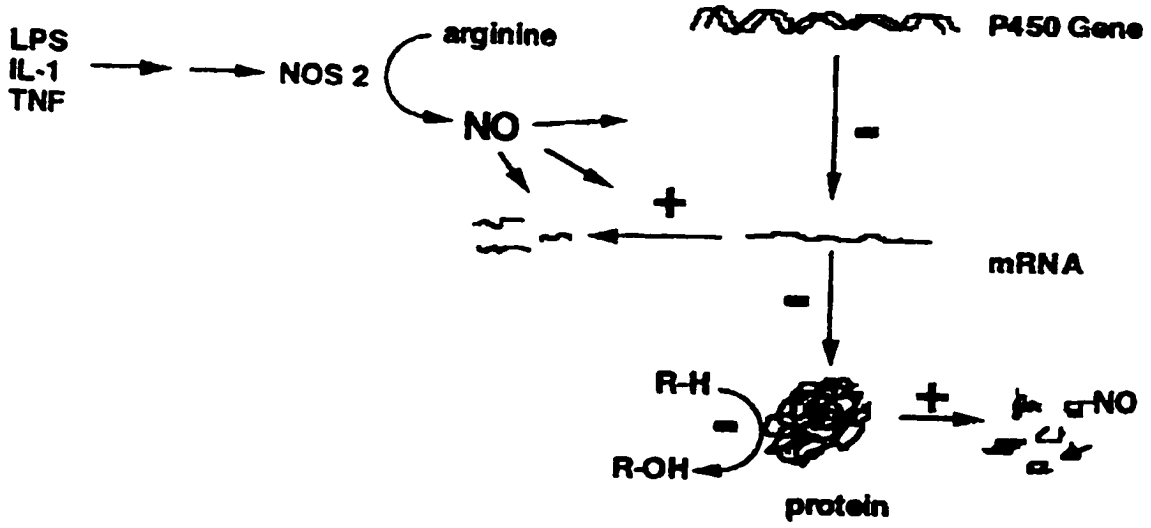
Cytokine effects on cytochrome P450 activity are very complex in nature with differential responses of isoforms to the major acute phase cytokines. The extent to which different isoforms of cytochrome P450 are affected by cytokines vary greatly indicating different modes of regulation for different isoforms of cytochrome P450 (Abdel-Razzak et al., 1993). It is in part for these reasons that much of the discrepancies seen between the isoforms affected in different models of infection or inflammation now seem to arise from the different subsets of cytokines released in these models (Muntané-Relat et al., 1995; Siewert et al., 2000).

1.4.4 NO and Cytochrome P450

NO is amongst the myriad of mediators released during infection or inflammation. As discussed previously, this molecule is a free radical and serves important immune

functions through its ability to destroy foreign pathogens (Lopez-Figueroa et al., 2000). One of the effects of NO is its ability to bind with the heme portion of guanylyl cyclase resulting in the stimulation of its activity. In a somewhat similar vein it has been suggested that NO may bind to the heme potion of cytochrome P450 and block the catalytic site resulting in a functional inhibition of the enzyme (Quaroni et al., 1996). Others have suggested that the interaction of NO with superoxide and the subsequent formation of the highly reactive peroxynitrite compound can nitrosylate the enzyme resulting in its inactivation and/or increased degradation (potential modes of cytochrome P450 regulation by NO are illustrated in figure 1-9) (Bolanos et al., 1997). Regardless of the mode of regulation, the effects of NO have been largely debated over the past 10 years. There is strong evidence for the participation of NO in the down-regulation of cytochrome P450 enzymes (Khatsenko et al., 1993; Wink et al., 1993; Stadler et al., 1994; Carlson and Billings, 1996; Müller et al., 1996; Khatsenko and Kikkawa, 1997; Khatsenko et al., 1998; Takemura et al., 1999) and equally convincingly evidence for the contrary (Hodgson and Renton, 1995; Sewer and Morgan, 1997; Sewer and Morgan, 1998). Although the various experimental models and/or the particular isoforms of cytochrome P450 examined likely contribute to this variability in results, recent evidence by Ferrari et al. (2001) suggests a dual mode of CYP2B1 regulation by LPS. At high concentrations of LPS (>0.1 μg) they report a NO-dependent decrease in CYP2B1 activity and protein at 24 hours. As well, they demonstrated that lower doses of LPS (<0.1 μg) decreased CYP2B1 mRNA in a NO-independent mechanism and resulted in decreased protein levels only after approximately 48 hours. Clearly then, the controversy

FIGURE 1-9



Potential Mechanisms for Cytochrome P450 Regulation by NO. NO may be involved in the downregulation of cytochrome P450 enzymes through various mechanisms such as inhibition of gene transcription, increased mRNA degradation, decreased mRNA stability, enhanced apoprotein degradation, or functional inhibition (taken from Iber et al., 1999).

surrounding the role of NO in the modulation of cytochrome P450 during infection and inflammation may be partially explained by comparing specific concentrations of LPS, incubation periods, and modes of regulation (activity, mRNA, protein).

1.4.5 Central Inflammation and Cytochrome P450

The clinical implications of compromised drug metabolism in patients with acute or chronic central inflammatory conditions such as MS, AIDS dementia, meningitis, Parkinson's, and Alzheimer's disease could be severe and far reaching as these patients typically receive numerous therapeutic agents for treatment. Altered drug handling could therefore not only result in potential toxicity but also in an impairment of drug function (specifically with pro-drugs). Shimamoto et al. (1998) administered LPS into the lateral ventricle of the brain as a model of bacterial meningitis. This treatment resulted in a substantial depression in the activity of the principle drug metabolizing isoforms of cytochrome P450 (CYP1A, 2B, 2C11, and 3A) in the liver. Renton et al. (1999) demonstrated that an i.c.v. injection of LPS had localized effects on the expression of CYP1A in the brain. In a later report we also showed that levels of cytochrome P450 (specifically CYP1A2) are depressed in the liver following administration of LPS into the brain (Renton and Nicholson, 2000). Both Shimamoto et al. (1999) and Renton and Nicholson (2000) demonstrated that the effects in the liver did not depend on activation of the HPA axis or sympathetic nerve stimulation. However, they did suggest cytokine release as a potential mechanism for these effects. Indeed substantial quantities of cytokines are often produced in response to CNS disorders and thus if they are

responsible for altering cytochrome P450 expression that would be a significantly important clinical concern for patients with these disorders.

1.5 Objectives of the Thesis

It is hypothesized that the effects of central inflammation on cytochrome P450 in the brain and liver are mediated by cytokines released in response to LPS. The main objectives centered on investigating this hypothesis are as follows:

1.) To establish an *in vitro* model in which to examine the direct effects of LPS on CYP1A activity in isolated glial cells.

2.) To investigate the potential role of immune mediators, released in response to LPS, in modulating CYP1A activity in glial cultures.

3.) To assess the role of cytokines in mediating the down-regulation of CYP1A activity in the brain during an experimentally induced central inflammatory response.

4.) To determine the role, if any, of cytokines as mediators of the decrease in CYP1A activity in liver during an experimentally induced central inflammatory response.

CHAPTER 2

GENERAL METHODS

2.1 MATERIALS

2.1.1 Animals:

All animals were obtained from Charles River Labs (Quebec, Canada) and housed in the Animal Care Facility of the Sir Charles Tupper Medical Building, Dalhousie University. Female Sprague-Dawley rats (14-15 days pregnant) were kept on standard bedding until the litters were delivered. Male Sprague-Dawley rats (125-150 g) were housed two per cage on clay chips in a 12-hour light/dark cycle and allowed free access to food and water. All animals were acclimatized to the facility for a minimum of 5 days prior to use.

2.1.2 Reagents

The materials used in the experiments described here are listed below. Common laboratory reagents are not included.

Sigma Chemical Company (St. Louis, MO)

Antifoam A

Anti-goat IgG conjugated to FITC

Anti-goat IgG conjugated to POD (peroxidase)

Anti-mouse conjugated to TRITC

Anti-rabbit IgG conjugated to biotin

Bovine serum albumin (BSA)

Butylated hydroxytoluene

Chlorzoxazone

Detoxified LPS (serotype 0127:B8)

Dibenz[*a*,*h*]anthracene

Dimethylsulfoxide

Enflurane

Ethoxyresorufin

Greiss Reagent

Lipopolysaccharide (serotype 0127:B8)

Monoclonal mouse anti-neurofilament 160

Nicotinamide adenine dinucleotide phosphate

Nitrocellulose

Pentoxyresorufin

Phenol Ciocalcateau reagent

Phenylmethyl Sulphonyl Fluoride

Pentoxifylline

Resorufin

Sodium dithionite

Sodium nitroprusside

Thimerosal

Tween 20

Triton X-100

Alexis Biotechnologies (San Diego, CA)

1400W

BDH (Toronto, Ont.)

Entellan

BioRad Laboratories (Hercules, CA)

Kaleidoscope polypeptide standards

Boehringer-Mannheim (Laval, PQ)

Monoclonal mouse anti-rat GFAP

CanSera (Ontario, Canada)

Fetal bovine serum

Cedarlane Labs Inc. (Hornby, Ont.)

TNF-α

IL-1β

Monoclonal mouse anti-rat CD11b/c (OX42)

Gentest (Woburn, MA)

6-hydoxychlorzoxazone

Monoclonal goat anti-rat CYP1A1

Polyclonal goat anti-rat CYP2E1

Polyclonal goat anti-rat CYP2B1

CYP1A and 2E1 protein standards

Life Technologies (Grand Lake, NY)

Antibiotic-antimycotic (100X)

Dublecco's modified eagles medium

Millipore (Bedford, MA)

Immobilon™-P (0.45 μm pore size)

R&D Systems (Minneapolis, MN)

IL-1α

IL-6

IFN-γ

StressGen Biotechnologies Corp. (Victoria, BC)

Polyclonal rabbit anti-mouse hsp25

Transduction Labs (Lexington, KY)

Polyclonal rabbit anti-mouse iNOS

Vector Laboratories, Inc. (Burlingame, CA)

3,3'-diaminobenzidine tetrahydrochloride

Horse anti-mouse IgG (rat absorbed) conjugated to biotin

Normal goat serum

Normal horse serum

Normal rabbit serum

Vectastain Elite ABC kit

2.2 *IN VITRO* METHODOLOGY

2.2.1 Isolation of Astrocytes:

Astrocytes were isolated from newborn Sprague-Dawley rats less than 24 hours old using an isolation procedure modified from that of Hertz et al. (1989). Briefly, brain tissue was removed from each rat and placed in cold serum-free DMEM containing antibiotic-antimycotic (100X containing 10 000 units of penicillin, 10 000 µg of streptomycin, and 25 µg of amphotericin B/ml). The tissue was homogenized by passage through a sterile 10 ml pipette twenty times then mixed vigorously in a vortex mixer for 1 minute to break-up cell aggregates and destroy neurons. The homogenate was then successively filtered through two nylon mesh bags (85 and 25 µm pore size) to remove cellular aggregates, blood vessels, and debris. The filtrate was centrifuged at 500 x g for 5 min. in a Beckman TJ-6 bench-top centrifuge (Beckman Instruments Inc., Fullerton, California), washed with sterile PBS and re-centrifuged. The cells were re-suspended in DMEM containing 20% FBS and antibiotic-antimycotic then plated in 5 ml culture dishes

using 1 ml of cell suspension and 4 ml of culture medium to give a final cell density of approximately $1.0x10^6$ cells/ml. In addition, cells were plated on 18 mm coverslips in 12 well plates using 0.5 ml of the cell suspension and 3.5 ml of culture medium.

Cultures were incubated at 37°C, 5% CO_2 and 95% air in a Sanyo MCO-17A CO_2 incubator (Sanyo Electric Company Inc., Japan) for seven days changing the medium three times per week. Following the seven-day incubation, the content of fetal bovine serum in the medium was reduced to 10% and the cells cultured for an additional seven days.

After the fourteen-day incubation period, monolayers of isolated cells cultured on the coverslips were used for histochemical analysis and plates of cells for enzymatic assays.

2.2.2 Cell Treatment:

Following the fourteen-day incubation period, the medium was removed from the cells and fresh DMEM (±10% FBS) was added along with antibiotic-antimycotic. CYP1A activity was induced by the addition of 50 nM DBA concurrently with the drug(s) of interest. Cells were incubated with these agents for a further 24-hour treatment period prior to assessment of enzymatic activity. Unless otherwise specified, in experiments where blockers were used, the cells were pre-treated with the blocker for 24 hours then the drug(s) of interest were added. Experiments were generally performed twice to ensure the specificity of the effect and the data shown are representative samples.

2.2.3 Ethoxyresorufin O-Dealkylase (EROD) Assay

CYP1A activity was determined by measuring the formation rate of resorufin from ethoxyresorufin using a modification of the procedure described by Burke et al. (1985). Cells were incubated with 0.6 μM ethoxyresorufin for 2 hours at 37°C then 2 ml of medium were removed from each plate. The fluorescence was measured in a Perkin Elmer Spectrofluorometer using an λ_{ex}=510 nm and an λ_{em}=586 nm. Determination of the fluorescence given by a standard concentration of resorufin (0.1 nmoles) allowed the calculation of the amount of resorufin formed in each sample. Results were expressed as the amount (pmoles) of resorufin formed per minute per milligram protein.

2.2.4 Protein Determination

Following the determination of EROD activity, the residual medium was removed, cells washed twice with PBS, then scraped into 2 ml of fresh PBS. Cells were lysed by sonication for 3 minutes then 250 μl of the cell sonicate was taken from each plate and protein content determined using the method of Lowry et al. (1951). Samples were diluted 1:4 in water then incubated with 2.5 ml of mixture 1 (1.97% $NaCO_3$ in 0.1 M NaOH, 0.02% sodium potassium tartrate, and 0.01% $CuSO_4$) at room temperature for 10 min. Phenol reagent (0.5 ml diluted 1:1 with water) was added to all samples, which were mixed and incubated at room temperature for 30 min. The absorbance of each sample (and duplicate) was measured at 700 nm using a Beckman DU-70 spectrometer (Beckman Instruments Inc., Fullerton, California). BSA was used as the standard and by averaging the duplicate values, protein content in the samples were calculated. In all cases, protein concentration is reported as mg/ml.

2.2.5 Immunofluorescence

The coverslips, containing a monolayer of astrocytes, were treated with drug(s) as described in section 2.2.2. Following the 24 hour incubation with drug, cells were washed several times in sterile PBS then fixed in 1 ml of cold methanol for 5 min. at -20°C. Cells were then double labelled for the astrocyte marker GFAP and CYP1A1/2 protein using a modification of the methods described by Montoliu et al. (1995). Briefly, cells were permeablized with 2% Triton X-100 and 3% hydrogen peroxide then blocked with 10% FBS in PBS. Coverslips were incubated overnight at 4°C with mouse anti-rat GFAP (1/500), goat anti-rat CYP1A (1/500), and 0.1% Evan's Blue to prevent autofluorescence. Cells were washed in PBS (3x 30 min.) then the secondary antibodies were added; anti-mouse IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC) (1/100) and anti-goat IgG conjugated to fluorescein isothiocyanate (FITC) (1/100) (refer to appendix A for complete listing of antibodies). Cells were incubated with secondary antibody for 1 hour at room temperature then washed five times for 30 minutes each with PBS and mounted on microscope slides. Fluorescence was visualized using a Nikon Eclipse 6800 fluorescent microscope (Nikon, Japan) with an excitation wavelength of 546 nm for FITC and 450-490 nm for TRITC. Images were obtained on Kodak 400 colour slide film with an exposure time of 30 seconds and a magnification of 40X. Slides were imaged using a Kodak SprintScan 35™ scanner and the software Adobe Photoshop 4.0™.

Using the same methodology as described above, cells were stained for anti-neurofilament by incubating cells with (1/40) dilution of the primary antibody (mouse

anti-neurofilament 160). Staining was visualized by using the secondary antibody anti-mouse IgG conjugated to TRITC. Fluorescence was visualized as described above.

2.2.6 Immunohistochemistry

Cells were grown on coverslips and prepared for staining as described in the previous section. The isolated astrocytes were stained for iNOS protein using a modification of standard techniques (Chao et al., 1994). Briefly, cells were permeabilized in PBST (PBS containing 0.1% Triton X-100) for 20 min. then incubated in 1% H_2O_2 to quench endogenous peroxidase. Cells were blocked with 2% goat serum in PBST for 1 hour at room temperature then exposed to an anti-mouse iNOS antibody that cross-reacts with rat iNOS (diluted 1:2500) for 16 hours at room temperature (refer to appendix A for complete listing of antibodies). Cells were exposed to a 1:200 dilution of a biotinylated goat anti-rabbit IgG secondary antibody then reacted with Advidin Biotin solution for 30 min at room temperature. Cells were reacted with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for approximately 3 min. and washed with PBS prior to mounting on glass slides. Images were obtained on an Axioplan 2 Zeiss microscope (Diagnostic Instruments Inc., USA) and processed using Adobe Photoshop 4.0™.

2.2.7 Cytokine Determination

Following administration of LPS, levels of TNF-α, IL-1β, and IFN-γ were measured using Quantikine® cytokine assay kits (R&D systems, Minneapolis, MN). Media samples were taken from treated cultures at 2, 4, 6, 12, and 24 hours following

drug addition. Samples were diluted 2-fold for TNF-α, 3-fold for IL-1β, and undiluted for IFN-γ. All samples plus standards were determined in duplicate using a colorimetric sandwich ELISA specific for the cytokine to be measured. Cytokine levels were determined by measuring the absorbance at 450 nm and using a standard curve to calculate the cytokine concentrations. Levels of cytokines are reported as the average concentration (ng/ml) per treatment at each time point. The limit of detection for each cytokine assay was 10 pg/ml for IFN-γ, 5 pg/ml for TNF-α and IL-1β.

2.2.8 Nitric Oxide Determination

Nitric oxide content was measured in culture medium by determining the amount of nitrite (NO_2^-) formed in each of the culture plates. Equal volumes of culture supernatant and Griess Reagent were added together and incubated at room temperature for 15 min. The absorbance of the resulting mixture was read at 550 nm and the amount of nitrite formed calculated using $NaNO_2$ as a standard. Results are expressed as nmoles NO_2^-/mg protein (Spitzer, 1994).

2.2.9 Western Blot

The cell sonicates for each treatment group (n=4) were pooled and the protein concentrated by centrifugation at 100 000 x g in a Beckman L-60 ultracentrifuge (Beckman Instruments Inc., Fullerton, California) for 45 minutes at 4°C (Chun et al., 1994). The pellets were re-suspended in brain buffer supplemented with 20% glycerol (0.1 M TRIS pH 7.4 containing 0.1 mM dithiothreitol, 0.1 mM EDTA, 1.15% KCl, 0.1 mM phenylmethylsuphonyl fluoride, 22 μM butylated hydroxytoluene, and 10%

glycerol). Samples were diluted by one-half with Laemmli buffer (0.12 M TRIS HCl, pH 6.8, 20% glycerol, 2% SDS, and 0.3 mM bromophenol blue) and separated by electrophoresis on a 7.5% ready-gel under non-reducing conditions (Bio-Rad, Hercules, CA) then electrophoretically transferred to a nitrocellulose membrane using a semi-dry transfer process (Tyler Research, Edmonton, Canada) (Smith, 1984). Blots were blocked in Blotto buffer (10% skim milk powder, 1% BSA, 0.01% Antifoam A, and 0.001% thimerosal) overnight at 4°C. Protein bands were detected immunologically using a goat anti-rat monoclonal antibody targeted against CYP1A1 that cross reacts with 1A2 (1/500 dilution in Blotto buffer) incubated overnight at 4°C with gentle agitation (refer to appendix A for complete listing of antibodies). Bands were visualized by chemiluminescence using an anti-goat IgG antibody conjugated to peroxidase (diluted 1/100 000 in Blotto buffer) and Supersignal Ultra chemiluminscent substrate (Pierce, Rockford, IL). Blots were exposed to Kodak X-OMAT x-ray film (Rochester, NY) and developed in a Kodak X-MAT M20 processor. Band intensities were measured with the Bio-Rad GS-690 imaging densitometer using the software Molecular Analyst™ and Scananalysis™. In all blots a set of molecular weight standards were used to determine the size of band detected and each blot contained a positive control, a CYP1A standard purified from 3-MC treated rats.

2.3 *IN VIVO* METHODOLOGY

2.3.1 I.c.v. Injections

Anaesthesia in animals was induced and maintained with enflurane (4%) throughout the surgical period. Animals were placed in a KOPF stereotaxic frame

(Tujunga, CA) and a longitudinal incision was made along the crown of the skull. The tissue was carefully dissected away until the bregma could be visualized. A burr hole was drilled through the skull +1.5 mm from the bregma. Intracerebroventricular injections (i.c.v.) were made by placing the needle tip 4.7 mm below the skull surface and slowly injecting the drug. LPS or cytokines were dissolved in sterile saline and injected in a 5 µl volume over a 1 min. period. After recovering from anaesthetic animals were allowed free access to water and food.

2.3.2 Tissue Isolation

Following the 24-hour recovery period, animals were killed by asphyxiation in a carbon dioxide chamber and the brain rapidly dissected out and rinsed in ice-cold brain buffer. The whole brain was homogenized in brain buffer (composition described in section 2.2.9) using a glass-teflon homogenizer then centrifuged at 850 x g for 10 min. in a Beckman TJ-6 bench-top centrifuge (Bhagwat et al., 1995). The supernatant was removed and centrifuged at 100 000 x g for 40 min. in a Beckman L-60 ultracentrifuge and the resulting pellet re-suspended in 400 µl of brain buffer supplemented with 20% glycerol. The membrane fractions were homogenized using a mini-glass/PTFE homogenizer then stored at -80°C.

The livers of these animals were also removed and rinsed in ice-cold KCl (1.15%) according to the methods of el-Masry et al. (1974). The tissues were homogenized using a Polytron (Brinkmann Instruments, Rexdale, Ont.) then centrifuged at 10 000 x g for 10 min. in a Beckman J2-21 centrifuge. The supernatant was removed and centrifuged in a Beckmann L-60 ultracentrifuge at 100 000 x g for 40 min. The resulting pellet was re-

suspended 50% by weight in glycerol buffer (50 mM KH_2PO_4, 20% glycerol, and 0.4% KCl) and homogenized in a glass homogenizer. Aliquots of the liver microsomes were stored at -80°C.

2.3.3 Protein Determination

The concentration of protein in tissue fractions from liver and brain were determined in an identical manner to that described in section 2.2.4. The sole difference being that the microsomes and brain membrane fractions were diluted 1:100 in the initial step rather than 1:4 as done with cells.

2.3.4 Hepatic P450 Content

Total hepatic P450 content was determined using the carbon monoxide (CO) difference spectrum assay as described by Omura and Sato (1962). Liver microsomes were diluted 1:4 in 1.15% KCl with 500 µl of 1 M KH_2PO_4 buffer and reduced with a small amount of sodium dithionite. The absorbance spectrums were measured then the samples were bubbled with CO for approx. 20 sec. and the absorbance difference spectrum (450/490 nm) was quantitated in a Beckman DU-70 spectrometer. P450 content was calculated ($\varepsilon = 91\ mM^{-1}cm^{-1}$) and reported as nmoles/mg protein.

2.3.5 Ethoxyresorufin O-Dealkylase (EROD) Assay

CYP1A (CYP1A1 and 1A2) activity was determined by the EROD assay as described in section 2.2.3. Isolated tissue (either hepatic microsomes or brain membrane fractions) was diluted 1:40 in 0.1 M KH_2PO_4 buffer (pH 7) and incubated with 0.5 µM

ethoxyresorufin for 2 min. at 37°C. The reaction was initiated by adding 10 µl of 25 µM NADPH to the incubation solution. The formation of resorufin, the fluorescent product was measured (λ_{ex}=510 nm; λ_{em}=586 nm) every 30 sec. for 2 min. Fluorescence of the standard (0.1 nmoles of resorufin) was then measured and used to calculate the rate of formation of resorufin per minute for each sample. Following protein determination, the EROD activity was normalized to the amount of protein present and expressed as pmol. resorufin/mg prot./min.

Control levels of activity tended to vary between experiments, therefore, separate control animals were used for each drug tested to ensure that the effects seen were solely due to the drug under investigation.

2.3.6 Pentoxyresorufin O-Dealkylase Assay

CYP2B activity was determined by measuring pentoxyresorufin O-dealkylase activity (PROD) using the methods of Burke et al. (1985). This assay was carried out in an identical manner to that described for the EROD assay with the following exceptions. The substrate for this assay was pentoxyresorufin (0.5 µM), the excitation wavelength was changed from 510 nm to 530 nm, and readings were taken every minute for 3 minutes.

2.3.7 Chlorzoxazone Hydroxylation Assay

CYP2E1 activity was determined in liver microsomes by measuring the rate of formation of 6-hydroxychlorzoxazone (6-OH CZX) from the parent compound chlorzoxazone (CZX) using a modification of published methods (Kharasch et al., 1993;

Tindberg et al., 1996). The reaction mixture (total volume = 1 ml), composed of 1 mg of microsomes and 0.1 mM CZX in 50 mM KH_2PO_4 buffer (pH 7), was allowed to equilibrate for 2 min. at 37°C. The reaction was initiated with the addition of 100 µl of 10 mM NADPH to the reaction mixtures of all samples except the blank. Samples were incubated for 10 min. at 37°C then the reaction was stopped by adding 5 ml of methylene chloride and 5 000 ng of the internal standard, pentoxifylline (PNTX). All samples were mixed vigorously for 15 min. then centrifuged at 500 x g in a Beckman TJ-6 centrifuge for 5 minutes. The organic phase was removed and evaporated down to dryness under a stream of nitrogen. Samples were re-suspended in 125 µl of mobile phase (17% acetonitrile:83% of 0.5% phosphoric acid) and 50 µl was injected on to a Beckman C_8 column (5 µm x 4.6 mm x 25 cm). Products were separated using a Waters 2690 pumping system and a Waters 2487 dual λ detector set at 287 nm. To facilitate the separation, a gradient method was used. The composition of the mobile phase was linearly increased from 17:83 to 25:75 and the flow rate was increased from 1.0 ml/min. to 1.5 ml/min. Using this method, the retention times for 6-OH CZX and PNTX were 11.1 and 13.5 min. respectively. The amount of product was calculated from a standard curve and the formation rate expressed as ng 6-OH CZX/mg protein/min.

2.3.8 Cytokine Determination

Following injection with LPS, or cytokines, levels of TNF-α, IL-1β, IL-6, and IFN-γ in blood were measured using Quantikine® cytokine assay kits (R&D systems, Minneapolis, MN). Peripheral blood samples were obtained from rats that were killed by CO_2 asphyxiation. Blood was collected from treated rats at 2, 4, 6, 12, or 24 hours

following i.c.v. injection, allowed to clot overnight at 4°C then centrifuged at 2 000 x g to obtain serum. Samples were diluted 2-fold for TNF-α and IL-6, 3-fold for IL-1β, and undiluted for IFN-γ. Cytokine levels were determined as outlined in section 2.2.7. Levels of cytokines are reported as the average concentration (ng/ml) in two animals per treatment at each time point. The limit of detection for each cytokine assay was 5 pg/ml for TNF-α and IL-1β and 10 pg/ml for IL-6 and IFN-γ.

2.3.9 Immunohistochemistry

Following the 24-hour treatment period, animals were deeply anaesthetized with pentobarbital (65 mg/ml) then perfused transcardically with 120 ml of 0.9% saline followed by 60 ml of 4% paraformaldehyde (PFA). Whole brains were removed and left in 4% PFA for 48 hours at 4°C then cryopreserved in 30% sucrose at 4°C until completely submerged. 40 μm coronal sections were cut on a Leica freezing microtome (Histoslide HM400, Canada) and stored in 1x Millonig's solution (made as a 10x concentrate: 0.75 M $NaH_2PO_4 \cdot 6H_2O$, 1 M NaOH, and 0.2 M NaN_3) until processed.

Heat shock protein 27 (hsp27) staining was carried out according to Garcion et al. (1998). Sections were washed with phosphate buffer (PB) (0.1 M $NaPO_4$, pH 7.4) for 30 min. then permeabilized with 3% hydrogen peroxide for 30 min. The sections were again washed (3x 15 min.) in PB then incubated overnight at 4°C with a rabbit anti-mouse hsp25 primary antibody diluted 1/5000 in 2% goat serum (Appendix A). Following treatment with primary antibody, sections were washed (3x 15 min.) then incubated with secondary antibody (biotinylated goat anti-rabbit IgG) diluted 1/400 in PB for 1 hour at room temperature. Sections were washed (3x 15 min.) and reacted with Advidin Biotin

solution for 60 min at room temperature then reacted with DAB and washed (3x 15 min.) with PB prior to mounting on glass slides. Tissue sections were mounted on glass slides, de-hydrated, and coverslipped in Entellan. Images were obtained on an Axioplan 2 Zeiss microscope and processed using Adobe Photoshop 4.0™.

Staining for microglia was carried out in a similar manner to that described above with the following modifications. Sections were permeabilized in 0.3% hydrogen peroxide for 30 min. then blocked with 2% horse serum in PB for 30 min. at room temperature. The primary antibody, diluted 1/10 000, was a mouse anti-rat CD11b/c (OX42) antibody (Appendix A). Brain sections were incubated with this antibody for 48 hr. at 4°C in PB with 2% horse serum then washed (3x 60 min.) with PB. Sections were incubated with secondary antibody (biotinylated horse anti-mouse, rat absorbed) diluted 1/400 with PB and incubated overnight at 4°C. With the exception of washing steps (3x 60min.), the remainder of the processing was carried out as described for hsp27.

2.3.10 Western Blot Analysis

All samples were diluted by one half with Laemmli buffer and contained 37.5 μg of protein. These samples were separated by electrophoresis on a 10% ready gel under non-reducing conditions (Smith, 1984). The resulting bands were electrophoretically transferred to an Immobilon P membrane using a wet transfer running overnight at 40 V (Bio-Rad, Hercules, CA). Membranes were blocked for 1 hour at room temperature with gentle agitation (1x TBS, 0.1% Tween20 with 5% w/v skim milk powder) then protein bands were detected immunologically according to standard procedures (Walker and Gaastra, 1988). Protein bands were detected using a monoclonal anti-rat CYP1A1

antibody (1/500 diluted in 1x TBS, 0.1% Tween20 with 1% BSA) that cross-reacts with CYP1A2 (Appendix A) or with polyclonal anti-rat CYP2E1 or CYP2B1 (same dilution). Bands were visualized using an anti-goat IgG antibody conjugated to peroxidase (1/100 000 diluted in blocking buffer) and Supersignal Ultra chemiluminescent substrate (Pierce, Rockford, IL). Blots were exposed to x-ray film (Hyperfilm™ ECL™, Amersham Pharmacia Biotech, England) for 3 min. then developed by hand (Kodak D-19 developer and a general purpose fixer). Band intensities were measured using the program Molecular Analyst™ (Bio-Rad, Hercules, CA). In all blots a set of molecular weight standards were used to determine the size of band detected and each blot contained a positive control where possible. Blots probed for CYP1A contained a standard purified from 3-MC treated rats and those examined for CYP2E1 contained a standard purified from ethanol treated rats.

2.4 Statistical Analysis

All data are reported as the mean ± S.E. (standard error of the mean). Single comparisons were made using an unpaired Student's T-test while multiple comparisons were made using the one-way analysis of variance (ANOVA) with significance determined by Student Newman-Keuls (Zar, 1974). In all cases, statistical significance was defined as having a p-value<0.05.

CHAPTER 3

MODULATION OF CYTOCHROME P450 BY INFLAMMATION IN ASTROCYTES

3.0 ABSTRACT

Activation of systemic host defense mechanisms results in the down-regulation of cytochrome P450 enzymes in the liver. This occurs for various induced and constitutive isoforms of cytochrome P450 in response to cytokines such as IFNs, IL-1, IL-6, and TNF-α, which are produced during infection. Although the levels of cytochrome P450 in brain regions are low, the enzymes are regionally distributed and may play critical roles in the activation or degradation of drugs and chemicals in localized areas. If activation of the immune response in the CNS by LPS modulates the activity of cytochrome P450 forms in the brain, this may alter normal metabolic pathways or contribute to drug or chemical toxicity.

This hypothesis was addressed by examining the effect of LPS on a major cytochrome P450 isoform in isolated astrocytes obtained from newborn rats. These cells were shown to express CYP1A when induced by dibenz[a,h]anthracene (DBA) as determined by enzyme activity, immunohistochemistry, and Western blotting. The treatment of these cells with LPS significantly attenuated the activity of these enzymes but had no effect on CYP1A1/2 protein levels as determined by Western blotting. The lack of effect by detoxified LPS indicates the requirement of the lipid A region on LPS to stimulate this response. Pentoxifylline (PNTX) prevented the LPS evoked decrease in CYP1A activity suggesting that cytokine release was a required component of this effect in astrocytes.

These results indicate that stimulation of the immune response by LPS in isolated astrocytes decreases CYP1A activity. The release of cytokines is implicated in this effect

and thought to participate in the functional inhibition of the enzyme as no effect on CYP1A1/2 protein levels was observed.

3.1 INTRODUCTION

Cytochrome P450 is a heme-containing superfamily of enzymes that metabolize a broad spectrum of endogenous and exogenous compounds (Gonzalez, 1990). Although primarily found in the liver, cytochrome P450 enzymes are expressed in many different tissues such as the lungs, kidney, skin, adrenal cortex, and nasal epithelium (Norris et al., 1996). In recent years, low levels of cytochrome P450 enzymes have been detected in brain tissue where the various isoforms are expressed in discrete regions of the brain (Norris et al., 1996; Riedl et al., 1996b; Hedlund et al., 1998). These enzymes are likely to be involved in diverse CNS functions including neurosteroid synthesis, metabolism of drugs, and the protection of brain tissue from toxins (Kapitulink et al., 1987; Anandatheerthavarada et al., 1990).

Infection and inflammation can modulate levels of cytochrome P450 enzymes in the liver both *in vivo* and *in vitro* leading to alterations in drug metabolism (Renton and Mannering, 1976b; Chang et al., 1978; Renton, 1981a; Muntané-Relat et al., 1995). In the systemic system, the immune response to either infection or inflammation is characterized by a release of cytokines that are necessary for the observed effects on cytochrome P450 enzymes (Ghezzi et al., 1986c; Paton and Renton, 1998). Administration of an immunogenic substance such as lipopolysaccharide (LPS) induces an inflammatory response in animals through the activation of macrophages and the subsequent release of cytokines (Paton and Renton, 1998). LPS administration has been shown to modulate cytochrome P450 enzymes in the liver both *in vivo* and *in vitro*, an effect that relies on the production of cytokines from stimulated macrophages (Ghezzi et al., 1986c; Paton and Renton, 1998).

Whether a similar phenomenon can occur with cytochrome P450 enzymes in the brain is still under active investigation. In comparison to the systemic system, the CNS immune response to a similar stimulus is much slower and less intense. Macrophages are recruited into the brain after a 48-hour delay while the recruitment of neutrophils is low or absent (Andersson et al., 1992). Some preliminary reports have shown that stimulation of an immune response or ischemic injury in the CNS can alter levels of cytochrome P450 enzyme expression (Tindberg et al., 1996; Zwain et al., 1997). These results may be critically important in many diseases of the brain such as Parkinson's disease, Alzheimer's disease, and MS that are thought to involve an inflammatory component (Hauss-Wegrzyniak et al., 1998).

In this report, we demonstrate that enzymatic activity of cytochrome P450 isoforms from the CYP1A subfamily (CYP1A1 and 1A2) are modulated by an LPS evoked inflammatory response in isolated astrocytes. Evidence is provided that cytokines participate in this response resulting in a functional type of inhibition of the enzyme.

3.2 MATERIALS AND METHODS

The preparation and treatment of cultures was carried out as described in chapter 2. Culture medium contained 10% FBS during all treatment periods in this chapter.

3.3 RESULTS

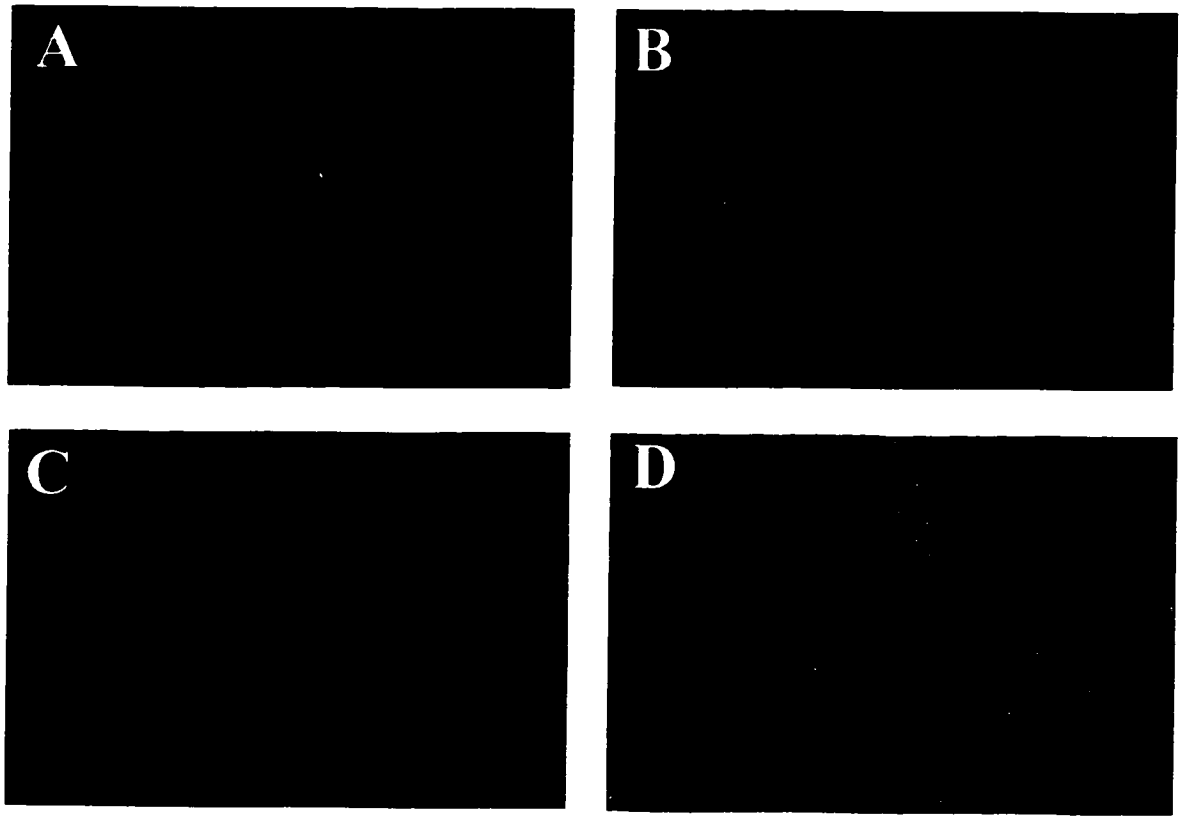
Immunohistochemical analysis of astrocyte cultures. Immunohistochemical studies using specific antibodies were carried out to confirm that isolated cells were astrocytes, demonstrate the absence of neurons, and to show that isolated astrocytes can express CYP1A protein. Immunoreactivity to an antibody directed against GFAP, a specific

astrocyte marker, indicated that the majority of isolated cells were astrocytes (Fig. 3-1A&C). When cells were probed with an antibody against neurofilament 160 no staining was observed demonstrating the lack of neuronal contamination (data not shown). The presence of CYP1A in control and DBA induced cells as indicated by immunoreactivity to an antibody against CYP1A is shown in figures 3-1B&D respectively. The level of CYP1A immunoreactivity in the DBA treated cells is considerably higher than in control cells. Double labeling of the cells for both GFAP and CYP1A1/2 demonstrated that cells staining positive for GFAP also stain for CYP1A confirming the expression in this cell type.

Effect of LPS on induced CYP1A activity. To study the effects of LPS on CYP1A forms, the enzyme was first induced by treatment with the aromatic hydrocarbon DBA. CYP1A activity was induced by approximately 25 times over vehicle controls (DMSO) in astrocytes that were incubated with 50 nM DBA for 24 hours. When 50 μg/ml LPS was added concurrently with 50 nM DBA to astrocytes and cultured for 24 hours, CYP1A activity was depressed by 50% relative to the corresponding control (Fig. 3-2). Western blot analysis demonstrated an increased level of CYP1A protein when cells were induced with DBA and similar, if not higher protein levels with concomitant administration of LPS (Fig. 3-3).

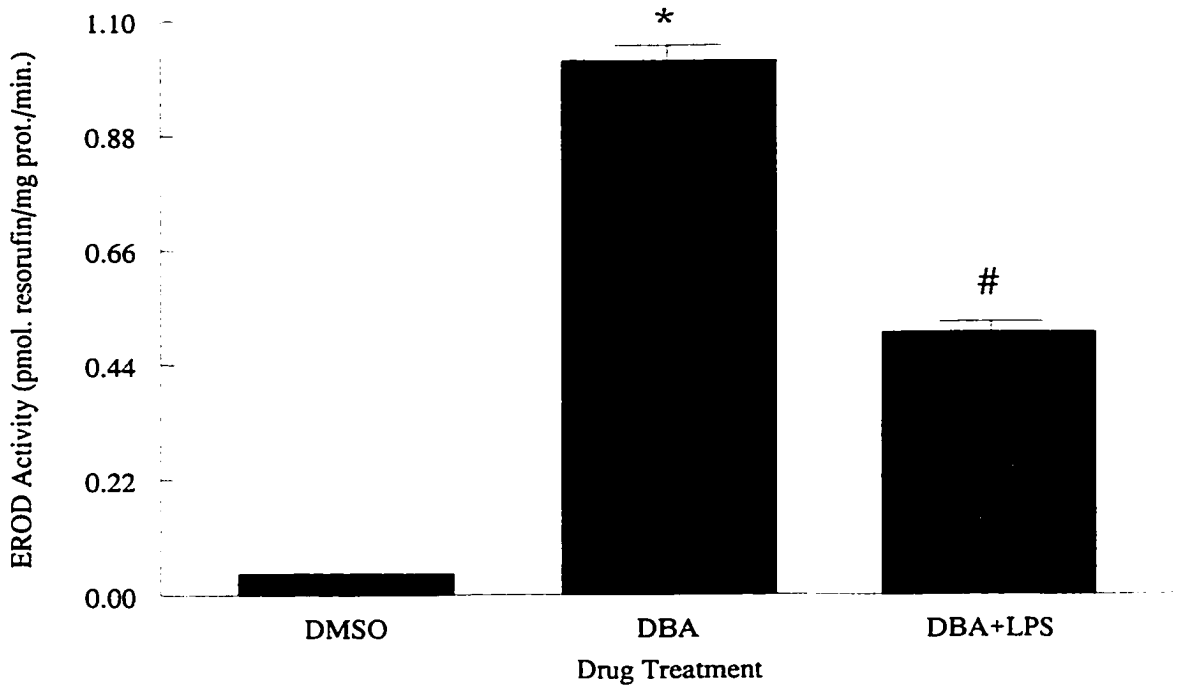
The effect of fetal bovine serum (FBS) on the ability of LPS to evoke a decrease in CYP1A activity was examined by comparing EROD activity in cells treated with LPS in both the presence and absence of serum. Cells treated with LPS in the presence FBS exhibited an 88% decrease in CYP1A activity compared with control levels (Fig. 3-4).

FIGURE 3-1



Immunofluorescence of Isolated Astrocytes. Expression of GFAP and CYP1A in astrocyte monolayers treated with DMSO **(A&B)** or with the inducing agent DBA **(C&D)**. Fluorescence double labeling of astrocytes was carried out using a monoclonal antibody against GFAP (1/500) with a TRITC-conjugated secondary antibody for visualization **(A&C)** and a monoclonal antibody towards CYP1A1 (1/500) with a FITC-conjugated secondary antibody **(B&D)**. Magnification = 40X

FIGURE 3-2



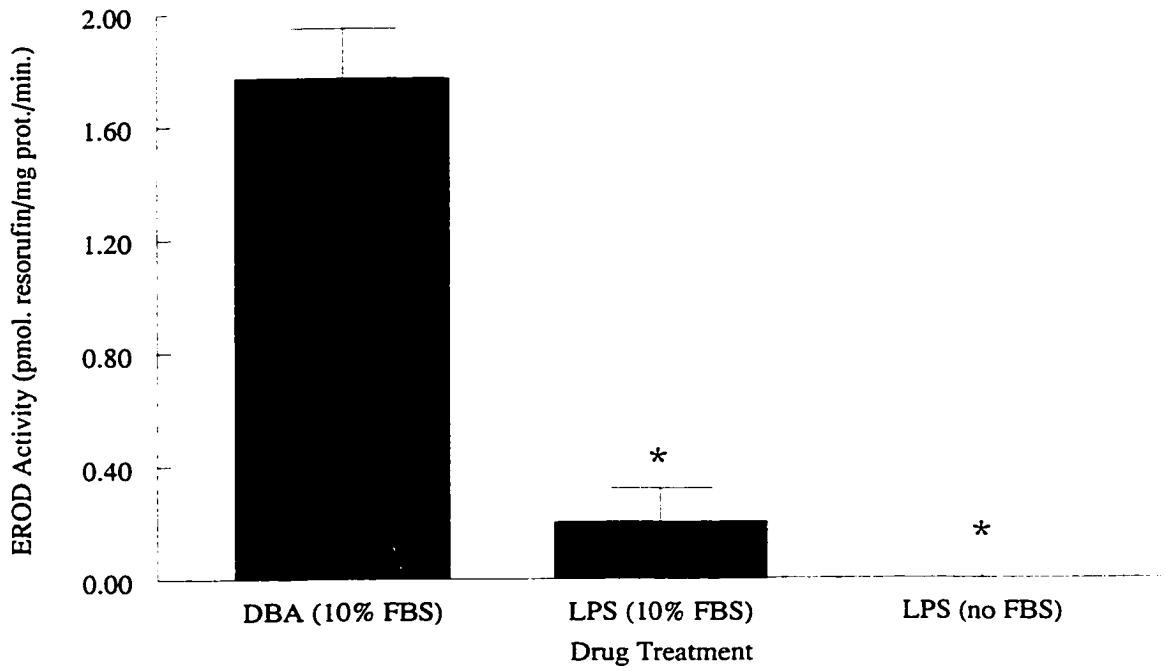
Ability of DBA to Induce CYP1A Activity in Cultured Astrocytes and its Suppression by LPS. Plated astrocytes were stimulated with 50 nM DBA in DMSO, the equivalent volume of vehicle, or 50 nM DBA concurrently with 50 μg/ml LPS for 24 hours (n=4 plates per treatment). Statistical analysis was carried out using a one-way ANOVA with statistical significance assessed by Student Newman-Keuls, $p<0.05$.

*Significantly different from vehicle.
#Significantly different with respect to DBA alone

FIGURE 3-3

The Effect of Drug Treatments on CYP1A1/2 Protein Levels in Astrocytes. Lanes 1-3 contain astrocytes treated with 50 μl of DMSO, 50 nM DBA, or 50 nM DBA+50 μg/ml LPS respectively. Each lane contains 7.5 μg of material pooled from the four individual plates of cells for each treatment group.

FIGURE 3-4



Effect of Fetal Bovine Serum on the Ability of LPS to Suppress Levels of CYP1A Activity in Cultured Astrocytes. Cells were treated with 50 nM DBA or 50 nM DBA+50 μg/ml LPS in the presence of serum or, 50 nM DBA+50 μg/ml LPS without serum (n=4 plates per treatment). EROD activity was measured 24 hours later and differences between treatments were assessed using the one-way ANOVA with Student Newman-Keuls to determine significance; $p<0.05$.

*Significantly different with respect to DBA alone

Similarly, in cells treated with LPS in the absence of FBS, CYP1A activity levels were completely abolished indicating that FBS was not necessary for LPS to mediate this effect. The induction of CYP1A by DBA was not affected by the presence or absence of FBS (data not shown).

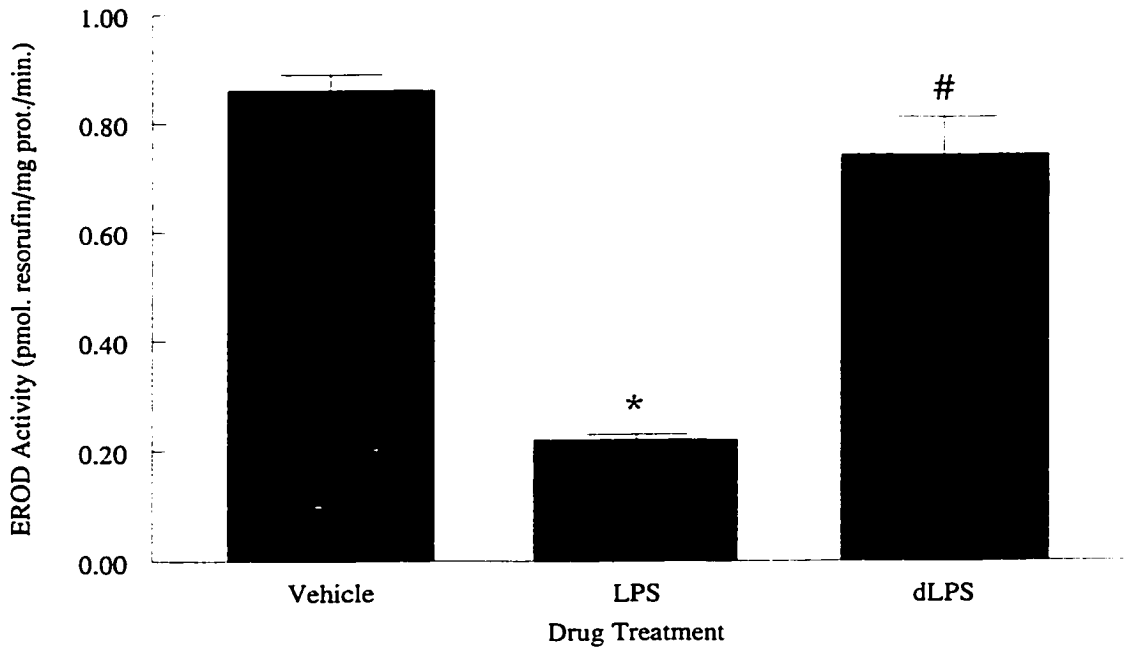
Detoxified LPS (dLPS) lacks the lipid A region, the area that is responsible for the immunogenic effects of this compound. When added to astrocytes, CYP1A activity was unaffected relative to control levels (Fig. 3-5). In comparison, astrocytes treated concurrently with LPS in the same experiment demonstrated a decrease in CYP1A activity compared to cells treated with dLPS or vehicle (saline). As seen with the previous experiment, Western blot analysis demonstrated that no substantial difference in the level of CYP1A protein was seen in astrocytes treated with LPS, dLPS, or DBA alone (Fig. 3-6A).

When added to astrocytes, the TNF-α synthesis inhibitor pentoxifylline (PNTX), prevented the decrease in CYP1A activity induced by LPS administration (Fig. 3-7). CYP1A activity in cells treated with LPS was decreased by 33% compared to controls whereas in cells treated concurrently with both LPS and PNTX, CYP1A activity was similar to controls. Protein levels, as judged by Western blot analysis were not affected by LPS or LPS + PNTX (Fig. 3-6B).

3.4 DISCUSSION

DBA induced the cytochrome P450 forms CYP1A1 and 1A2 in cultured astrocytes as demonstrated by the immunohistochemical studies, an enzymatic activity assay (EROD), and Western blot analysis of protein levels. This treatment provided a

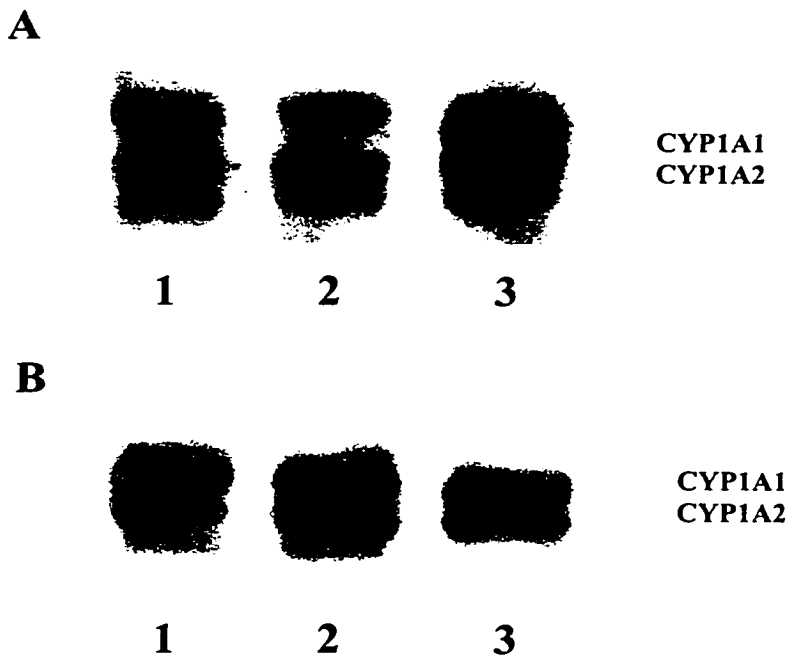
FIGURE 3-5



Effect of Detoxified LPS on Levels of CYP1A Activity in Isolated Cells. EROD activity was determined in astrocytes treated with 50 nM DBA + saline (vehicle), 50 nM DBA+50 μg/ml LPS (LPS), or 50 nM DBA+50 μg/ml dLPS (dLPS) for 24 hours (n=4 plates per treatment). Differences between drug treatments were assessed using a one-way ANOVA with Student Newman-Keuls; $p<0.05$.

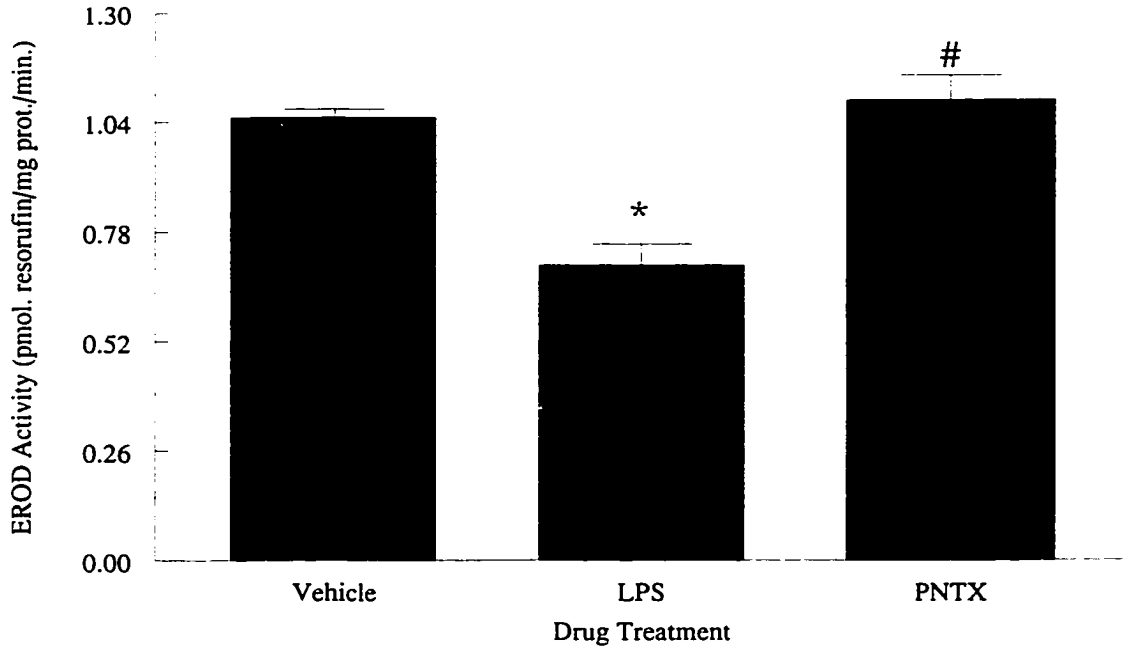
*Significantly different with respect to vehicle control
#Significantly different with respect to LPS treatment

FIGURE 3-6



Representative Western Blots for Astrocytes Treated with dLPS or LPS in Combination with PNTX. Each lane contains 7.5 μg of material pooled from the four individual plates of cells for each treatment group and were separated out on a 7.5% ready gel under non-reducing conditions. **(A)** The effect of dLPS on CYP1A1/2 protein levels in astrocytes treated with 50 nM DBA+vehicle, 50 nM DBA+50 μg/ml LPS, or 50 nM DBA+50 μg/ml dLPS (lanes 1 through 3 respectively). **(B)** The effect of PNTX on levels of CYP1A1/2 protein in astrocytes treated with 50 nM DBA+vehicle, 50 nM DBA+50 μg/ml LPS, or 50 nM DBA+50 μg/ml LPS+87 μM PNTX (lanes 1 through 3 respectively).

FIGURE 3-7



PNTX Evoked Reversal of the Suppression in CYP1A Activity by LPS. EROD activity was determined in astrocytes treated with 50 nM DBA+saline (vehicle), 50 nM DBA+50 μg/ml LPS (LPS), or 50 nM DBA+50 μg/ml LPS+87 μM PNTX (PNTX) for 24 hours (n=4 plates per treatment). Statistical significance was determined using a one-way ANOVA and Student Newman-Keuls; $p<0.05$

*Significantly different with respect to DBA alone
#Significantly different with respect to LPS treatment

sufficient level of enzyme to determine if LPS could modulate the activity of these isoforms. The expression of CYP1A is distributed in specific areas of the brain including the striatum, hypothalamus, globus pallidus (CYP1A1), and olfactory bulb (CYP1A2) (Riedl et al., 1996b). These enzymes metabolize compounds such as caffeine, aflatoxin B, heterocyclic arylamines and they may play an important role in the protection of neurons from potentially toxic compounds. It is therefore necessary to determine whether the expression of these enzymes can be modulated by inflammation in the CNS. The results presented here clearly demonstrate that LPS is capable of suppressing CYP1A activity without a concomitant decrease in the corresponding protein levels. The lack of an effect on protein levels by LPS implicates a post-transcriptional type of regulation such as enzyme inhibition. It is possible that this inhibitory effect is mediated through the release of cytokines and the subsequent generation of free radicals such as NO. NO is thought to be able to bind to the heme portion of the cytochrome P450 enzyme and block the active site thus resulting in a functional type of enzymatic inhibition as seen here (Wink et al., 1993). The results described here are not in agreement with what was found by Tindberg et al. (1996) who found a decrease in CYP1A1 protein following a 24 hour exposure to 100 ng/ml LPS. This discrepancy may result from the fact that the astrocytes used in the current experiments were induced with DBA. Another potential explanation may arise from differential modes of regulation seen with low concentrations of LPS such as that used by Tindberg et al. vs. the higher concentrations used in the experiments described here (Ferrari et al., 2001).

In an earlier study, Paton and Renton (1998) showed that LPS did not lower cytochrome P450 activity directly in a hepatoma cell line (Hepa1) but required the

presence of macrophages to evoke the response. It was postulated that this resulted from the lack of CD14 expression in Hepa1 cells however, macrophages do express this receptor, which, upon activation can evoke the release of cytokines. Astrocytes, like Hepa1 cells do not seem to contain the requisite CD14 receptors necessary for release of cytokines in response to LPS administration. However, in contrast to liver cells, LPS is reported to induce the expression of CD14 receptors on astrocytes rendering them susceptible to the direct effects of endotoxin as evidenced by the significant decrease in CYP1A activity in these cells (Lacroix et al., 1998). LPS binds through its lipid A region to a high affinity serum binding protein termed the lipopolysaccharide binding protein (LBP). Binding of LPS to LBP facilitates the activation of membrane bound CD14 receptors on immunocompetent cells that in turn stimulate the synthesis and release of cytokines such as IL-1, IL-6, and TNF-α. In addition to membrane bound CD14 receptors, the LPS-LBP complex can also bind to soluble CD14 receptors which in turn can stimulate CD14 negative cells to synthesize and release cytokines (Kielian and Blecha, 1995). In the experiments reported here, LPS was thought to mediate its effect through binding to LBP and the CD14 receptors on cells. However, in the absence of serum, LPS was still capable of decreasing CYP1A activity. This implied that LPS was not dependent upon stimulation of the CD14 receptor via opsinization with LBP. Rather, it is possible that LPS exerts its effects through another low affinity type of receptor or via direct activation of the CD14 receptor (Kielian and Blecha, 1995). This could account for the relatively large concentration of LPS required to elicit an effect in these experiments (50 μg/ml vs. nanogram quantities for the high affinity pathway). The LPS induced suppression of CYP1A activity is mediated through the interaction of LPS with a

specific receptor as evidenced by the lack of effect of detoxified LPS that does not contain the lipid A region; this form of LPS did not attenuate CYP1A activity.

Another possible mechanism for the LPS induced down-regulation of CYP1A could be through activation of microglia that express CD14 receptors. Although the majority of isolated cells stained positive for the astrocyte marker GFAP, the preparation used in these studies may contain some residual microglial cells. These cells, as well as astrocytes, release cytokines in response to LPS administration (Frei et al., 1987; Van Dam et al., 1992; Willis and Nisen, 1995). IL-1 and TNF-α from microglial cells activated by LPS may contribute towards the loss of CYP1A activity in astrocytes.

The interaction of LPS with its receptor induces the synthesis and release of cytokines, which are responsible for the effects of LPS both *in vivo* and *in vitro*. The prevention of LPS induced suppression of CYP1A by PNTX suggests that cytokine release is a required step in the down-regulation of CYP1A, as it is known to inhibit the synthesis of a variety of cytokines (D'Hellencourt et al., 1996).

In liver, the decrease in cytochrome P450 enzyme activity induced by immunostimulation requires the release of cytokines such as TNF-α, IL-1α, IL-1β, IL-6, or the IFNs (Ghezzi et al., 1986c; Stanley et al., 1988; Poüs et al., 1990; Barker et al., 1992; Abdel-Razzak et al., 1993; Fukuda et al., 1994; Muntané-Relat et al., 1995; Paton and Renton, 1998). It was therefore hypothesized that a similar event may occur in brain to account for the observed results. Frei et al. (1987) demonstrated that mouse microglial cultures released TNF in response to LPS, as judged by the ability of anti-TNF antibodies to block the effect. Astrocytes participate in the brain immune response by their ability to secrete IL-1 in response to immunostimulants such as LPS (Van Dam et al., 1992).

These observations support the idea that cytochrome P450 enzymes located within brain tissue may be modulated by immunostimulation via the release of cytokines. At the concentration used, PNTX is a fairly specific inhibitor of TNF-α synthesis, thus demonstrating the role of this cytokine in mediating LPS evoked suppression of CYP1A.

In summary, the results reported in this paper indicate that LPS can down-regulate CYP1A in CNS derived cells and implicates TNF-α in addition to other cytokines as mediators of this effect. The observation that LPS has a direct effect on astrocytes whereas in liver, the presence of macrophages is required suggests that astrocytes have the ability to produce and respond to cytokines in an autocrine fashion (Zwain et al., 1997).

CHAPTER 4

THE ROLE OF CYTOKINES IN THE DEPRESSION OF CYP1A ACTIVITY USING CULTURED ASTROCYTES AS AN *IN VITRO* MODEL OF CENTRAL INFLAMMATION

4.0 ABSTRACT

The modulation of hepatic cytochrome P450 enzymes by infection and inflammation has been well described both in clinical settings and in animal models. Recent evidence found that central inflammation leads to alterations in cytochrome P450 activity in both the brain and in liver. The bacterial endotoxin LPS was utilized to induce an inflammatory response in cultured astrocytes as a model of CNS inflammation. This inflammatory response involves a range of immune mediators such as acute phase cytokines, nitric oxide, prostanoid products and reactive oxygen species. It is hypothesized that cytokines, released during inflammation, act to modulate the expression of specific isoforms of cytochrome P450 resulting in altered activity levels. High levels of the cytokines TNF-α and IL-1β were released into culture medium following the addition of LPS to astrocyte cultures. When these same cytokines were added directly to the cultures they also were able to modulate levels of CYP1A activity. The concurrent addition of dexamethasone to astrocytes blocked both the cytokine release and the alteration of CYP1A activity induced by LPS, thus supporting a role for these cytokines in this response. These results provide evidence suggesting an involvement of acute phase cytokines in mediating the LPS induced depression of CYP1A activity in cultured astrocytes.

4.1 INTRODUCTION

Cytochrome P450 is a superfamily of enzymes that are well known for their role in the metabolism and excretion of drugs from the body. These enzymes are primarily but not exclusively found in the liver and adrenal glands as they are also distributed throughout the body in diverse areas such as the intestine, skin, lungs, and brain (Norris et al., 1996). Cytochrome P450 content in the brain is approximately 0.5-3% of the content in liver and thus should not significantly contribute to overall drug elimination. However, these enzymes have been shown to be highly localized in discrete areas within the brain parenchyma and thus may alter the local actions or concentrations of neuroactive drugs (Majewska et al., 1986). In addition, it has been shown that cytochromes P450 in the brain may have homeostatic functions as isoforms have been shown to participate in cerebral blood vessel tone and also in the synthesis of neuroactive steroids (Walther et al., 1987b; Warner et al., 1994; Harder et al., 1997). Current evidence shows that CYP1A1/2, CYP2B1, CYP2E1, CYP2D1, novel forms from the CYP3A and CYP4F families, and CYP7B exist in the brain and are regionally located in both neuronal and glial cells (Strobel et al., 1995; Miksys et al., 2000).

Recent evidence from our laboratory and others has shown that several cytochrome P450 isoforms found in brain are depressed during a localized CNS inflammatory response induced by an i.c.v. injection of LPS (Shimamoto et al., 1998; Renton et al., 1999; Renton and Nicholson, 2000; Nicholson and Renton, 2001c). The mechanism by which LPS evoked inflammation causes a depression in CYP1A activity has not been elucidated, although it is likely to involve mediators generated from immunocompetent cells within the brain parenchyma during inflammation (Perry et al., 1993; Montero-Menei et al., 1996). In response to an immune stimulus, glial cells,

specifically astrocytes and microglia become activated and stimulate the acute phase response. This process is characterized, in part, by the release of immune mediators including cytokines, prostanoid products, and NO (Rivest et al., 2000).

In a previous report from this laboratory we demonstrated that glial cells harvested from rat brain tissue are predominately astrocytes (approx. 95%) and it is these astrocytes that are capable of expressing the cytochrome P450 isoforms CYP1A1/2 in response to the chemical inducer DBA. Cultured astrocytes have been used as an *in vitro* model for assessing the local effects of immune-modulators on cytochrome P450 activity in CNS derived cells (Tindberg et al., 1996; Nicholson and Renton, 1999). In this section we examined the role of cytokines in mediating the down regulation of cytochrome P450 in CNS derived cells.

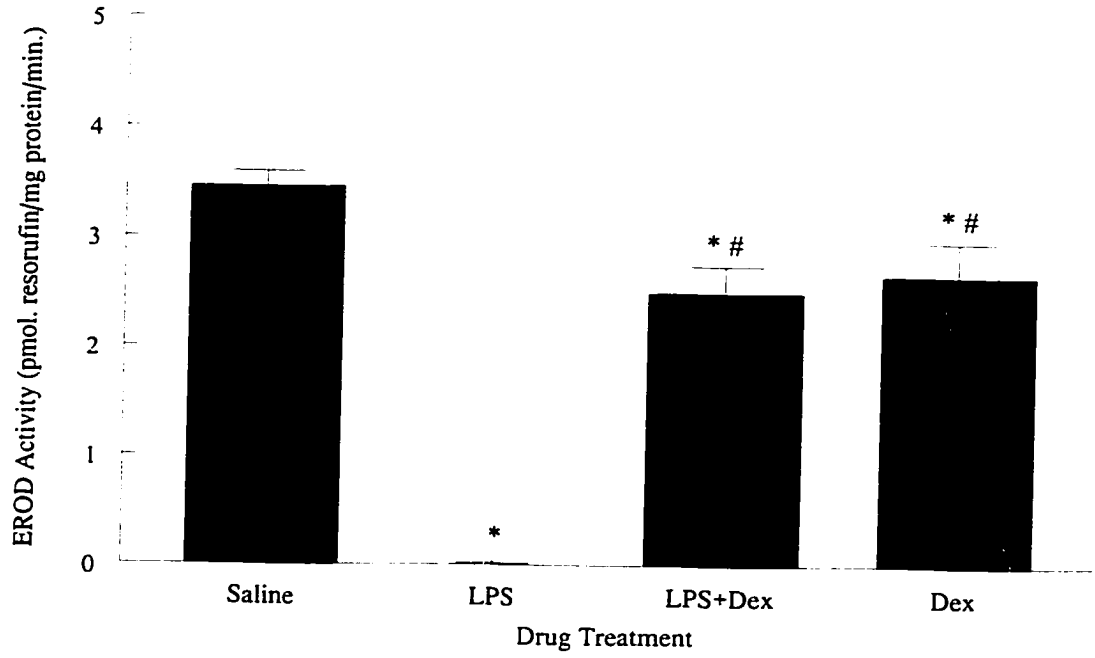
4.2 MATERIALS and METHODS

The isolation protocol, treatment of cultures, and enzymatic assays were carried out as described in chapter 2. Drug treatments were carried out in serum-free medium and thus a reduced concentration of LPS (25 μg/ml) was used. All astrocytes cultures contained 50 nM DBA to induce CYP1A activity.

4.3 RESULTS

Effect of LPS on induced CYP1A activity. The addition of LPS (25 μg/ml) to cells for a 24-hour period resulted in a complete loss of CYP1A activity as indicated by EROD activity (Fig. 4-1). When cells were pretreated with the cytokine synthesis inhibitor, dexamethasone (DEX) (40 μg/ml), the addition of LPS to the cells resulted in a decrease in EROD activity of only 28%.

FIGURE 4-1



The Effect of DEX on the LPS Induced Decrease in CYP1A Activity in Cultured Astrocytes. Cells were pretreated with a 40 μg/ml concentration of DEX for 30 min. prior to the addition of 50 nM DBA and 25 μg/ml LPS. Astrocytes were incubated with drugs for 24 hours before measuring EROD activity (n=4 plates per treatment). Statistical significance was determined using a one-way ANOVA with Student Newman-Keuls ($p<0.05$).

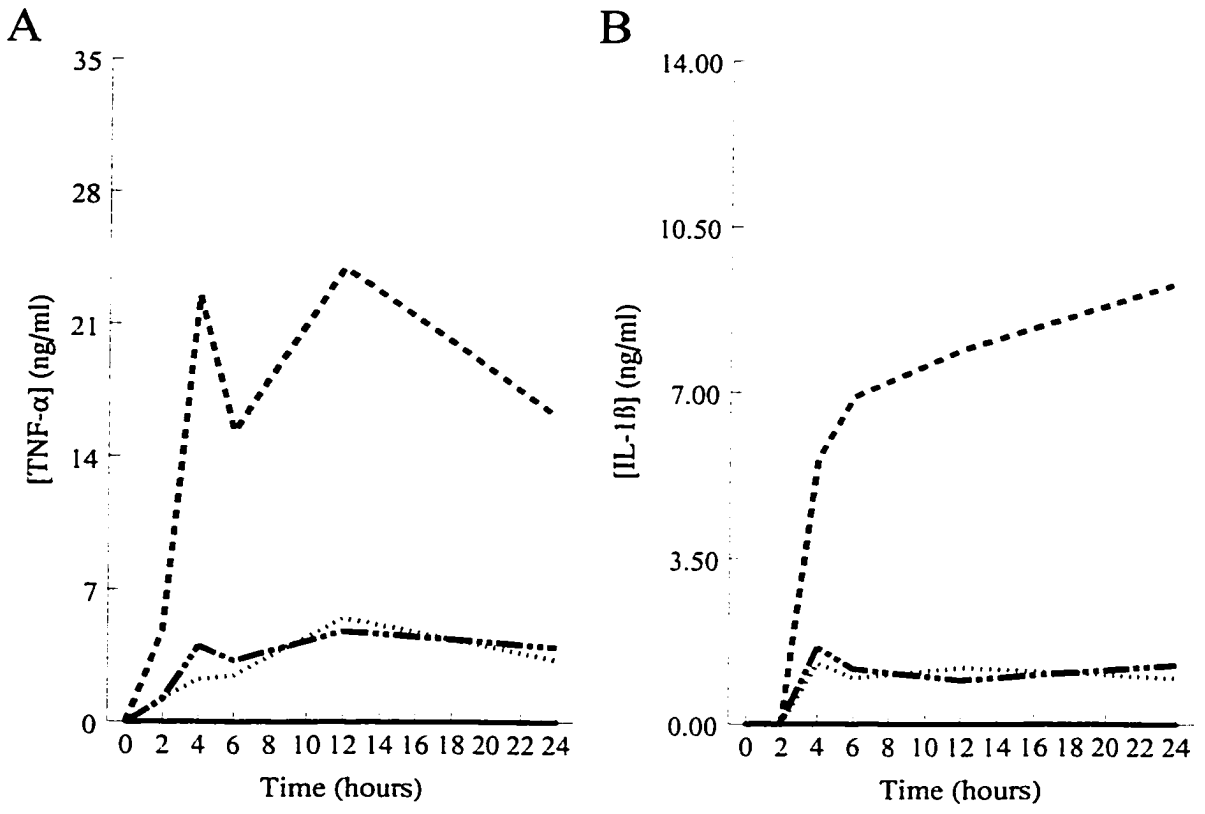
*significant with respect to saline control
#significant with respect to LPS treated

This observed decrease was identical to the depression occurring in cells treated with DEX alone (Fig. 4-1).

LPS induced cytokine release in cultured astrocytes. Following the incubation of astrocytes with 25 μg/ml of LPS, samples of culture medium were obtained at 2, 4, 6, 12, and 24 hours to assess levels of cytokine release during the culture period. These cells responded to LPS by the production of TNF-α within two hours and the levels remained elevated throughout the culture period (Fig. 4-2A). Similarly, LPS treated cells produced IL-1β within 4 hours and the levels were sustained throughout the 24-hour period (Fig. 4-2B). No detectable TNF-α or IL-1β was produced in cells treated with an equivalent volume of saline. The addition of DEX to the cultures significantly reduced the production of TNF-α and IL-1β stimulated by LPS (Fig. 4-2A&B). The levels of cytokines released from cells treated with LPS and DEX were identical to levels observed in cells treated with DEX only. In contrast to TNF-α and IL-1β, LPS was unable to induce IFN-γ release in any of the cell treatments at any time point (data not shown).

Cytokines modulate CYP1A activity in astrocytes. Several acute phase cytokines were assessed for their ability to modulate CYP1A activity when added directly to cultured astrocytes (Fig. 4-3). At a concentration of 20 ng/ml, TNF-α caused a 38% decrease in EROD activity. Incubation of astrocytes with IL-1β lowered EROD activity by 23% at a concentration of 15 ng/ml. In contrast, IL-6 (28 ng/ml) and IL-1α (9 ng/ml) had no effect on EROD activity when added to astrocytes cultures (Fig. 4-3). In cells treated with IFN-

FIGURE 4-2



The LPS Induced Release of Cytokines from Cultured Astrocytes. Samples of media were taken from individual plates at 2, 4, 6, 12, and 24 hours following LPS exposure and assayed for levels of cytokine. Each determination was done in duplicate and the values averaged. **(A)** Levels of TNF-α and **(B)** IL-1β found in the samples of medium.

FIGURE 4-3

The Effect of Cytokines on CYP1A Activity in Cultured Astrocytes. Cultured astrocytes were treated with various cytokines for 24 hours. In each case the solid bars represent the control levels of EROD activity and the hollow bars the activity seen in treated cultures. Each treatment represents separate experiments in which several concentrations of cytokines were examined. The graphs illustrated here are representative of the effects seen in these experiments (n=4 plates per treatment). Drug treatments shown here are 20 ng/ml TNF-α, 15 ng/ml IL-1β, 28 ng/ml IL-6, and 9 ng/ml IL-1α and had corresponding control activity levels of 5.01±0.49, 2.74±0.11, 3.12±0.52, and 1.38±0.14 pmol. resorufin/mg protein/min. respectively. Statistical significance was assessed on individual experiments using a one-way ANOVA with Student Newman-Keuls ($p<0.05$).

*significantly different from the corresponding control.

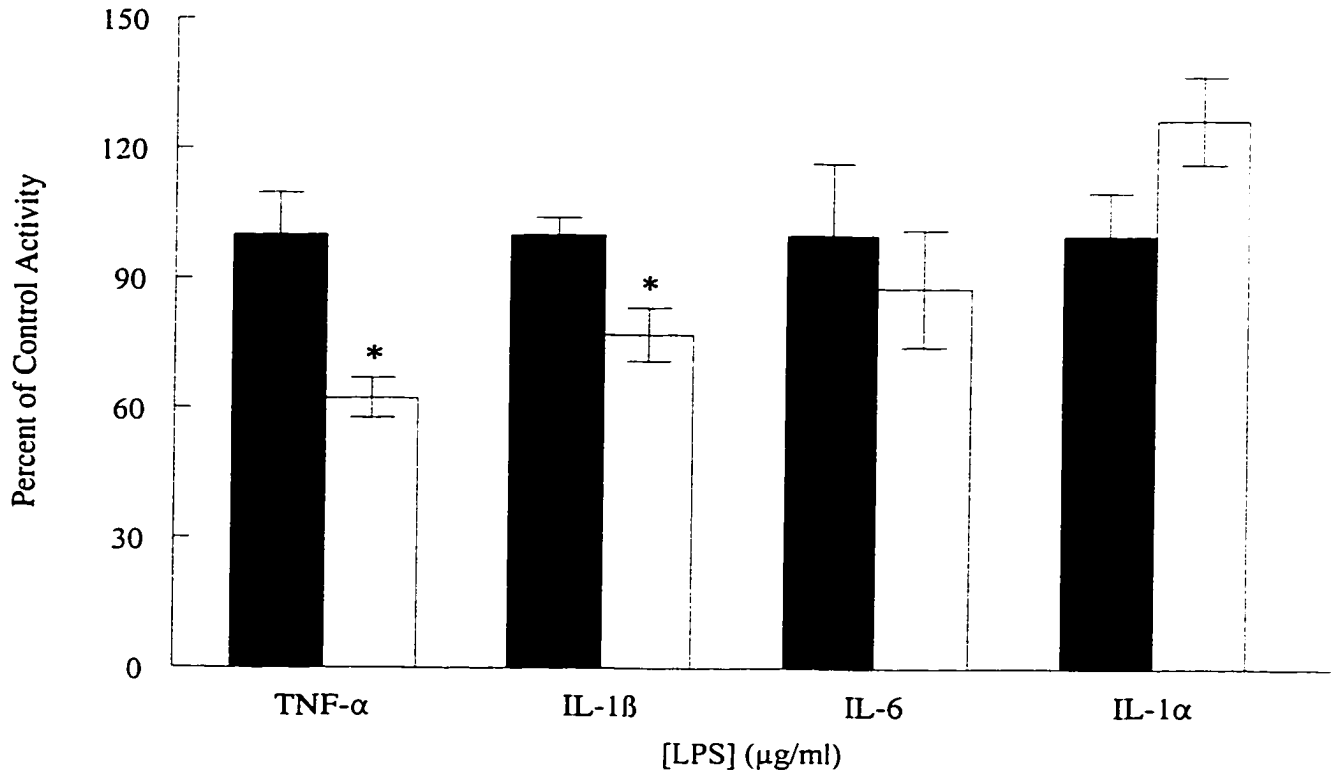

150
120
90
60
30
0
Percent of Control Activity
*
*
TNF-α
IL-1ß
IL-6
IL-1α
[LPS] (μg/ml)

FIGURE 4-3

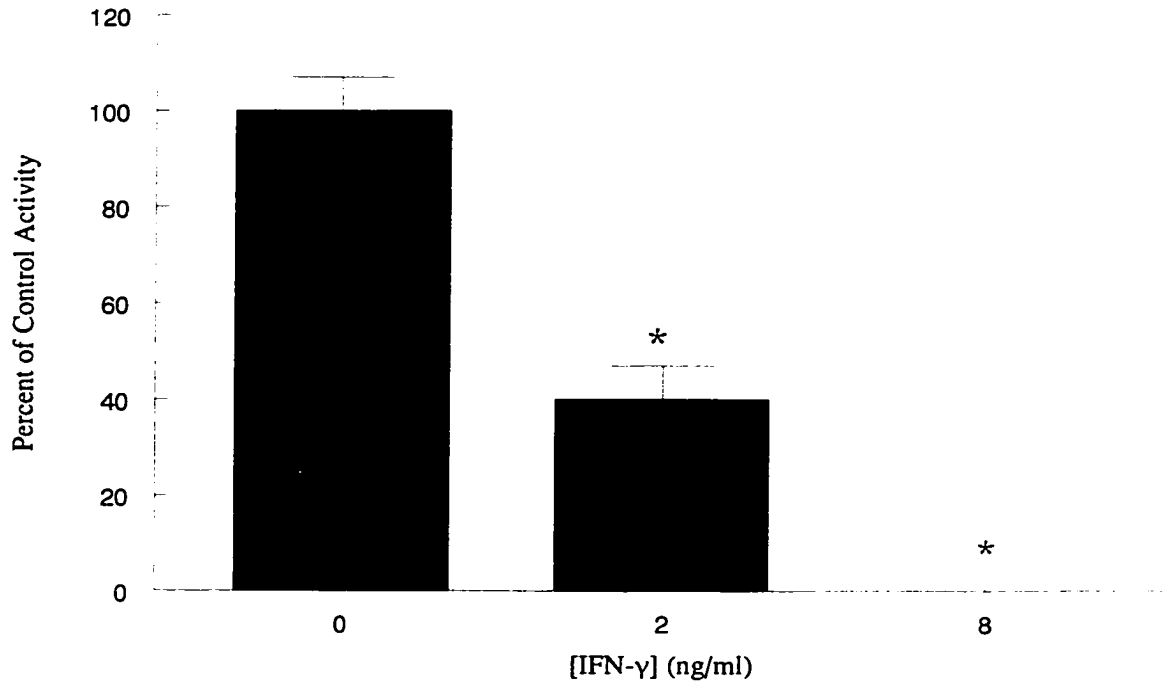
γ, a concentration-dependent decrease in EROD activity occurred (Fig. 4-4). IFN-γ decreased CYP1A activity significantly by 60 and 100% at concentrations of 2 and 8 ng/ml respectively.

Nitric oxide release induced by Immunostimulants. NO levels were determined in culture medium of cells treated with LPS or cytokines. As shown in table 4-1, LPS induced the release of substantial amounts of NO whereas the cytokines TNF-α, and IFN-γ did not release detectable amounts of NO. In the case of IL-1β, a concentration of 15 ng/ml, which is sufficient to depress CYP1A activity, did not release detectable amounts of NO, but a concentration of 50 ng/ml resulted in the production of 15.8 ± 1.5 nmol. NO_2^-/mg protein.

4.4 DISCUSSION

The administration of LPS to both animals and humans is known to modulate the activity of a variety of cytochrome P450 enzymes in both peripheral organs and in the brain (Shedlofsky et al., 1994; Morgan, 1997; Renton et al., 1999; Renton and Nicholson, 2000). When added to isolated astrocytes, LPS has been shown to alter the activity of CYP1A1/2 and CYP2E1 isoforms (Tindberg et al., 1996; Nicholson and Renton, 1999). In this report we utilized cultured astrocytes as a model system to investigate the role of cytokines in the LPS evoked decrease in CYP1A activity in the brain. The results reported in this section demonstrate that CNS derived cells respond to an immune stimulus (LPS) by the release of acute phase cytokines. These cytokines may be potential mediators of the decrease in CYP1A activity in the brain that has been reported during a

FIGURE 4-4



The Effect of IFN-γ on CYP1A Activity in Astrocytes. Cells were treated with either 2 or 8 ng/ml IFN-γ for 24 hours then the EROD activity was assessed (n=4 plates per treatment). The control activity level was determined to be 2.15±0.25 pmol. resorufin/mg protein/min. Statistical significance was determined using the one-way ANOVA with Student Newman-Keuls ($p<0.05$).

*significantly different with respect to control.

TABLE 4-1

NO release following incubation with immune stimulants (n=4 plates per treatment).

Drug Treatment	**[NO] (nmoles nitrite/mg protein)**
Saline	Not detectible
25 μg/ml LPS	142.3 ± 9.7
20 ng/ml TNF-α	Not detectible
15 ng/ml IL-1β	Not detectible
50 ng/ml IL-1β	15.8 ± 1.5

localized inflammatory reaction (Nicholson and Renton, 2001c).

The effects of LPS on hepatic cytochrome P450 activity have been well established and characterized. Several lines of research have convincingly demonstrated that these effects result from the stimulation of immune responses in the periphery. Ghezzi et al. (1986c) performed serum transfer experiments in a strain of LPS resistant mice to demonstrate that LPS itself did not directly mediate the depression in cytochrome P450 but that a serum mediator, specifically IL-1, was the critical mediator. Paton and Renton (1998) demonstrated that hepatoma cells did not respond to LPS directly, but when incubated with medium from macrophages stimulated by LPS, they responded by decreasing CYP1A activity. In comparison to the periphery, the brain has a much subtler immune response, which is activated by insults such as brain trauma and CNS infections (Matyszak, 1998). This immune response is characterized by the activation of astrocytes and microglia in a process termed gliosis (Andersson et al., 1992). This results in the generation of several intermediate products such as acute phase cytokines, NO via the increased expression of inducible nitric oxide synthase, and stimulation of the arachidonic acid cascade (Gottschall et al., 1992; Matyszak, 1998; Lopez-Figueroa et al., 2000).

Many of the effects of LPS (peripherally and centrally) are known to occur through the generation of intermediate products such as cytokines, proteases, free radicals, and prostaglandins (Kielian and Blecha, 1995; Montero-Menei et al., 1996; Paludan, 2000). It is likely that the effect of LPS on CYP1A activity in the model described here occurs through the stimulation of astrocytes and the subsequent generation these types of intermediate products.

The synthetic glucocorticoid DEX, is a commonly used anti-inflammatory drug that prevents cytokine synthesis by activating the transcriptional inhibitor Iκ-B (Scheinman et al., 1995). When primary cultures of astrocytes were incubated with DEX concurrently with LPS there was a complete prevention of the LPS induced decrease in CYP1A activity. In addition, DEX dramatically decreased the release of two major acute phase cytokines, TNF-α and IL-1β normally released following incubation with LPS. These observations support the idea that acute phase cytokines could mediate the effects of LPS on CYP1A activity in astrocytes. Incubation of cultured astrocytes with acute phase cytokines such as TNF-α or IL-1β in a concentration similar to that produced by cells in response to LPS, could partially mimic the effects of LPS and may contribute to the effects of LPS in astrocytes. This also supports the idea that LPS decreases CYP1A activity in astrocytes via cytokine production. IFN-γ dramatically decreased CYP1A activity in cultured astrocytes implicating it as a potential mediator of these effects. However, IFN-γ could not be detected in medium from cultured cells stimulated by LPS. This result was surprising as IFN-γ is released *in vivo* following LPS administration (Ho, 1964; Nicholson and Renton, 2001c). It appears that cultured glial cells, although very sensitive to this cytokine, do not release it in response to LPS. Thus, IFN-γ is unlikely to contribute to the effects of LPS on CYP1A activity in cultured astrocytes.

In contrast, no effect of IL-6 or IL-1α was observed on CYP1A activity. The effects of IL-6 on cytochrome P450 activity have been widely reported but are contrasting in nature. Earlier work using *in vitro* models has shown IL-6 to be capable of depressing the activity of a variety of cytochrome P450 isoforms (Fukuda et al., 1992; Fukuda et al., 1994). However, the effects of IL-6 become less clear when administered

in vivo as it has a variety of different effects depending on the isoform examined (Morgan, 1991; Morgan et al., 1994). IL-6, at any concentration examined, did not affect CYP1A activity in isolated astrocytes indicating that this cytokine does not appear to mediate the effects of LPS in this model. It is also surprising that IL-1α had no effect on CYP1A activity in astrocytes as the biological activities of both IL-1 isoforms are mediated through the same receptor and IL-1β depressed CYP1A activity (Anforth et al., 1998). Earlier work *in vivo* also showed an inability of IL-1α to modulate CYP1A activity in brain whole membrane fractions (Nicholson and Renton, 2001c). In addition, it has been reported that the relative potencies of these two isoforms of IL-1 are not necessarily identical (Anforth et al., 1998). Of the major acute phase cytokines released during inflammation, it has become apparent that at least two of these cytokines, IL-1β and TNF-α can be implicated in the LPS evoked decrease of CYP1A in cultured astrocytes.

Astrocytes respond to an immune challenge by the synthesis and release of immune mediators such as the acute phase cytokines (Sawada et al., 1989; Chung and Benveniste, 1990). These cytokines in turn can act in an autocrine or paracrine manner to affect a decrease in CYP1A activity. In the cellular make-up of the glial cultures utilized here the vast majority of cells (95%) are astrocytes with a minor contribution of microglia (3%) and oligodendrocytes (2%). It is possible that cytokines, produced by activated microglia may participate in the loss of CYP1A activity in astrocytes. However, the contribution of cytokines released from this cell type is likely to be relatively minor as the percentage of microglia in this culture system is fairly small.

Another potential mediator that may be involved in the loss of CYP1A by LPS is NO, which is released by LPS in these cultures. It has been well established that substantial quantities of NO are released during inflammation and in response to immune stimuli such as LPS (Boje and Arora, 1992; Chao et al., 1992; Zielasek et al., 1992). It has been reported that cytokines induce the release of NO from immunocompetent cells, and thus it is possible that NO, released by astrocytes, is responsible for part, or all of the effects of cytokines on CYP1A activity. However, the levels of NO produced in astrocytes treated with cytokines were very low or negligible. Measurable amounts of NO are only detectable following treatment of cultures with 50 ng/ml IL-1β whereas suppression in CYP1A activity in these cultures occurred at a much lower concentration (15 ng/ml) that produced undetectable levels of NO. These observations suggest that the actions of acute phase cytokines on CYP1A activity in astrocyte cultures do not involve an NO-dependent mechanism.

These results implicate cytokines as playing a major role in the LPS induced decrease in CYP1A activity in cultured astrocytes. As neither TNF-α nor IL-1β could fully mimic the effects of LPS on CYP1A activity, it is likely that other mediators might work in consort with them to cause this down regulation in activity.

CHAPTER 5

THE ROLE OF CYTOKINES IN THE LIPOPOLYSACCHARIDE EVOKED DEPRESSION OF CYTOCHROME P450 IN THE BRAIN AND LIVER

5.0 ABSTRACT

A role for cytokines as mediators of the depression in cytochrome P450 activity in brain and liver during CNS inflammation is proposed. Lipopolysaccharide (LPS) was given directly into the lateral ventricle of the brain to mimic a localized CNS infection. CYP1A activity and protein in both brain and liver were depressed in response to this treatment. The administration of the pro-inflammatory cytokines TNF-α, IL-1β, and IFN-γ directly into the lateral ventricle emulated the effects of LPS on CYP1A activity only in the brain. In contrast, these centrally administered cytokines did not produce a concomitant loss of CYP1A2 activity in the liver.

Significant levels of several cytokines (TNF-α, IL-1β, and IFN-γ) were produced in the serum of animals following i.c.v. LPS administration. This production of peripheral cytokines by LPS could not be mimicked by the administration of IL-1β nor TNF-α by i.c.v. injection.

These results suggest that induction of cytokines in the brain may play a direct role in the depression of CYP1A activity in the CNS following the administration of LPS into the lateral ventricle. The production of cytokines within the brain does not appear to participate in the signaling process in the brain that leads to the concomitant loss of CYP1A2 activity in the liver. The subsequent production of cytokines in peripheral tissues does however, appear to play a role in the loss of cytochrome P450 in the liver.

5.1 INTRODUCTION

Cytochrome P450 enzymes are known for their role in the metabolism of a vast array of endogenous and exogenous compounds. Although the majority of isoforms reside in the liver, they are known to occur in extra-hepatic tissues such as the adrenal glands, lungs, kidneys, skin, and brain (Norris et al., 1996). Within the CNS, expression of specific cytochrome P450 isoforms tends to be low, unequally distributed, and regionally localized (Schilter and Omiecinski, 1993; Norris et al., 1996; Riedl et al., 1996a; Hedlund et al., 1998). The localization of enzymes in the brain parenchyma and in circumventricular organs are thought to contribute to a number of important functions including the local synthesis of neurosteroids, participation in blood vessel integrity, and potentially could play a role in chemical toxicity (Kapitulink et al., 1987; Anandatheerthavarada et al., 1990).

Hepatic cytochrome P450 and drug biotransformation are susceptible to modulation by viral and/or bacterial infections via the activation of an immune or inflammatory response and subsequent generation of cytokines (Chang et al., 1978; Ghezzi et al., 1986c; Paton and Renton, 1998). Recently, a similar response has been described for the effects of inflammation on cytochrome P450 in the CNS (Renton et al., 1999; Renton and Nicholson, 2000). In the CNS, immune activation is a much subtler process than that which occurs in the periphery. Immune activation caused by agents such as the bacterial endotoxin LPS results in microglial activation and the recruitment of monocytes from the periphery into the brain (Montero-Menei et al., 1996). These macrophages, microglia, and astrocytes become activated and release a cascade of inflammatory cytokines including TNF-α, IL-1β, and IL-6 all of which mediate the

subsequent immune response in the CNS (Perry et al., 1993). In addition to the release of cytokines, the metabolism of arachidonic acid is stimulated and the expression of inducible nitric oxide synthase occurs (Boje and Arora, 1992; Sehic et al., 1996). We have recently demonstrated that central inflammation induced by i.c.v. administration of LPS not only depressed CYP1A activity in the brain but also produced a concomitant loss of the enzyme in the liver (Renton et al., 1999; Renton and Nicholson, 2000). The mechanisms involved in the loss of cytochrome P450 in the brain and liver in response to CNS inflammation remains unclear.

Many neuro-degenerative disorders such as Parkinson's or Alzheimer's disease are thought to involve a chronic inflammatory component within the brain (Hauss-Wegrzyniak et al., 1998; McGeer and McGeer, 1999). We have hypothesized that such conditions may produce cytokines in response to CNS inflammation and alter the level of cytochrome P450 activity and/or expression within the brain and liver. This may result in aberrant drug handling or alter the production or degradation of endogenous compounds. In this section we examine the role of cytokines in the loss of cytochrome P450 in brain and liver during an inflammatory response confined to the brain.

5.2 MATERIALS and METHODS

The tissue preparations and enzymatic assays were carried out as described in chapter 2.

5.3 RESULTS

The effect of LPS on CYP1A activity in the brain and liver. The injection of LPS (25 μg) into the lateral ventricle evoked a significant decrease in CYP1A activity in whole

brain membrane fractions as shown in figure 5-1A. EROD activity, a measure of CYP1A activity, was depressed by 35% following a 24-hour exposure to the bacterial endotoxin. Western blot analysis indicated a significant decrease in CYP1A protein in the brains of treated animals compared to controls (Fig. 5-2A). The detoxified version of LPS (dLPS) cannot elicit an immune response as the lipid A portion of the molecule has been stripped off and thus cannot activate CD14 receptors on immunocompetent cells (Chensue et al., 1991). When administered into the lateral ventricle, dLPS had no significant effect on brain EROD activity (Fig. 5-1A).

Liver microsomes obtained from these animals were examined for alterations in the activity and expression of hepatic CYP1A2. EROD activity was significantly decreased (36%) 24 hours after the injection of LPS into the lateral ventricle (Fig. 5-1B). Western blot analysis of these microsomes demonstrated that hepatic CYP1A2 protein levels were significantly decreased by 69% in response to an i.c.v. injection of LPS (Fig. 5-2B). EROD activity in the liver of animals treated with dLPS remained unchanged following the 24-hour treatment period (Fig. 5-1B).

The effect of cytokines on CYP1A activity in brain and liver. The inflammatory cytokines TNF-α, IL-1β, and IFN-γ were examined as potential mediators of the LPS-induced depression of CYP1A activity. TNF-α (5 ng) injected i.c.v., decreased the level of EROD activity in brain by 27% but there was no concomitant effect on EROD activity in the liver (Fig. 5-3A&B). Similarly, a 44% decrease in brain EROD activity occurred in response to 2.5 ng of IL-1β administered by i.c.v. injection with no concomitant effect on liver EROD activity (Fig. 5-3A&B). As well, no difference in EROD activity in the

FIGURE 5-1

The Effect of LPS and dLPS on CYP1A Activity in Brain and Liver. 25 μg of LPS or dLPS was given as a single dose into the lateral ventricle of the rat and the EROD activity examined 24 hours later. Each drug treatment represents an individual experiment with the solid bars representing the saline controls and the clear bars, the treated animals (LPS or dLPS) (n=4 animals per treatment). **(A)** EROD activity in brain membrane fractions (saline control = 0.34±0.01 pmol. resorufin/mg prot./min. for the LPS experiment and 0.27±0.02 pmol. resorufin/mg prot./min. for the saline control in the dLPS experiment). **(B)** EROD activities in liver microsomes (saline control = 57.6±3.5 pmol. resorufin/mg of LPS experiment and 96.1±10.5 pmol. resorufin/mg prot./min. for the dLPS experiment. Statistical significance was determined using an unpaired Student's t-test.

*significant with respect to corresponding saline control.

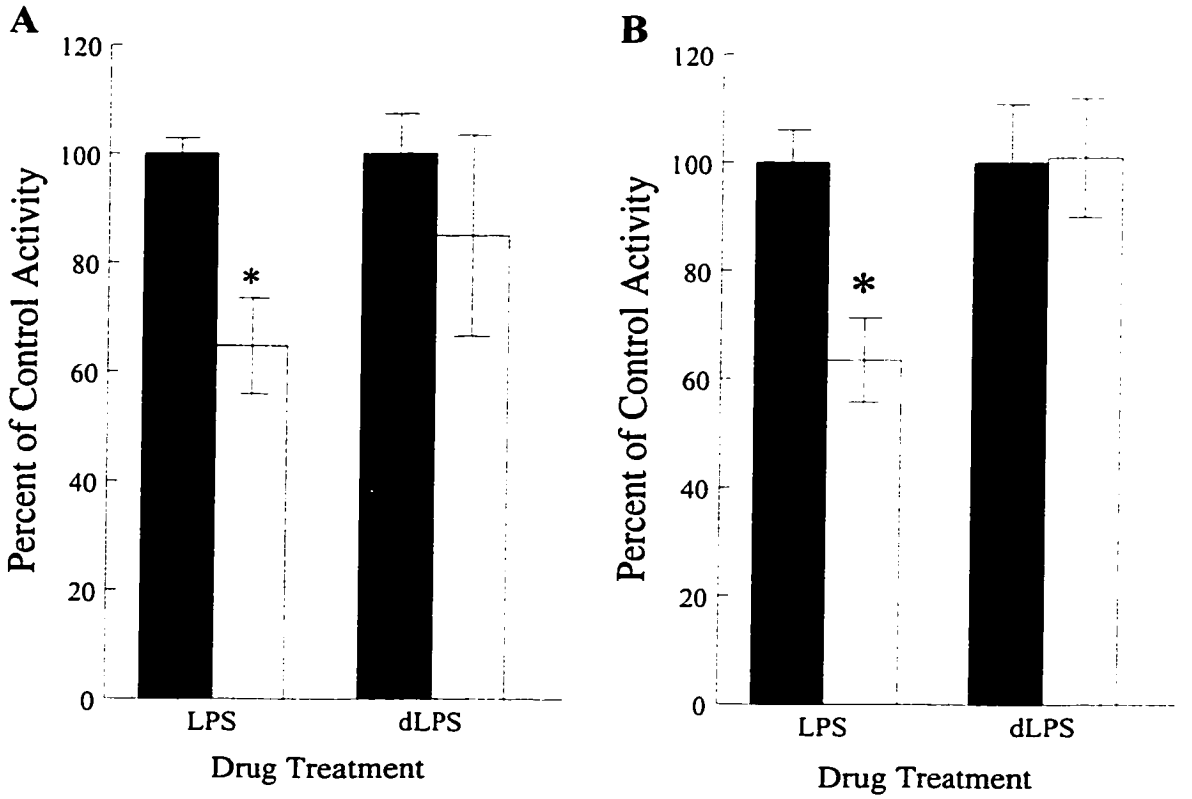
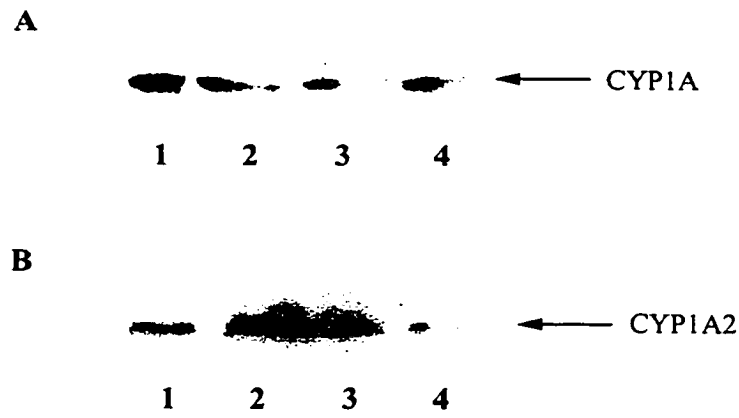

A
120
100
80
60
40
20
0
Percent of Control Activity
*
LPS
dLPS
Drug Treatment
B
120
100
80
60
40
20
0
Percent of Control Activity
*
LPS
dLPS
Drug Treatment

FIGURE 5-1

FIGURE 5-2

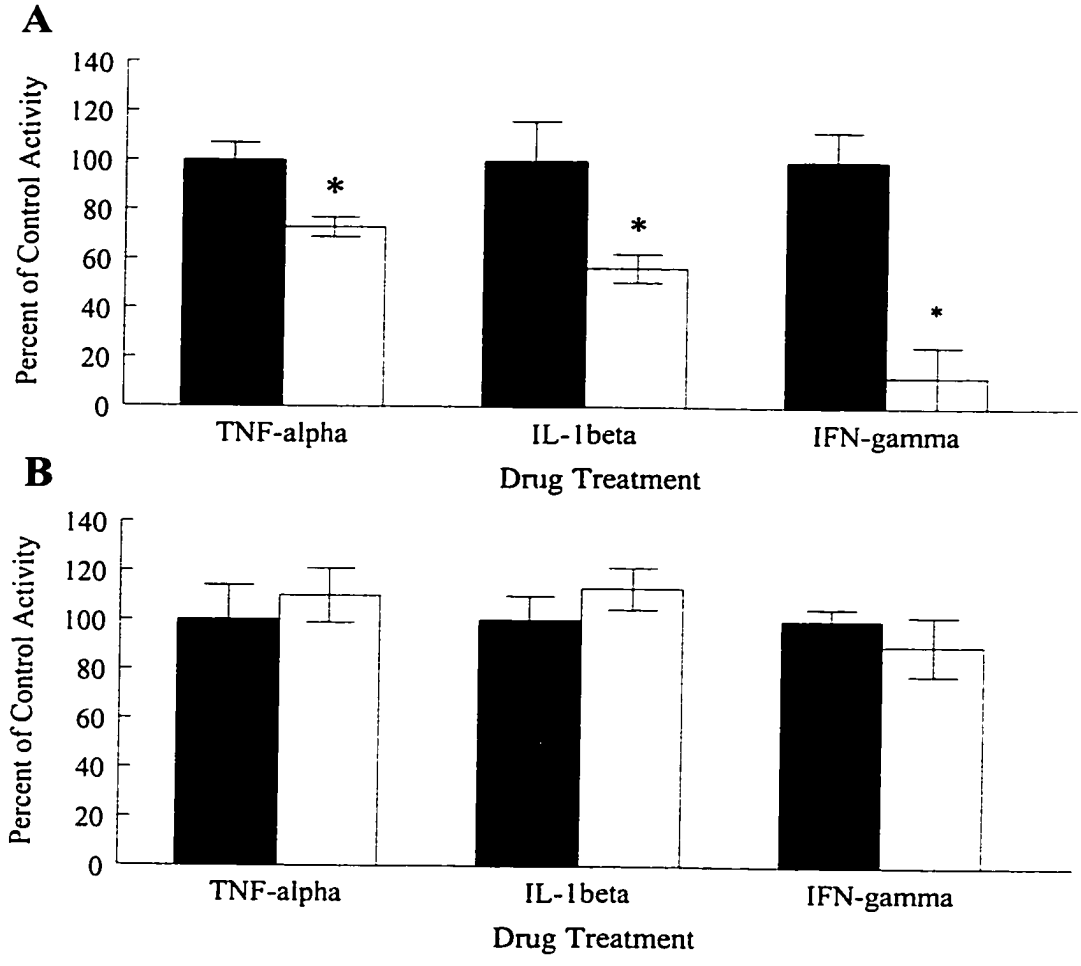


Effect of LPS Given by i.c.v. Injection on CYP1A Protein in Brain and Liver Tissue. In these representative Western blots, each lane contains 37.5 μg of protein from each rat. **(A)** Lanes 1&2 are brain tissue from control animals and lane 3&4 animals treated with 25 μg LPS (i.c.v.). CYP1A apoprotein levels in brain were significantly decreased in treated animals (642±116 vs. 293.8±8.0 arbitrary units for saline and LPS respectively) (n=4 animals per treatment). **(B)** Lanes 1&2 are liver tissues from saline controls and lanes 3&4 are LPS treated animals. CYP1A2 apoprotein levels in liver were significantly different in animals that received LPS by i.c.v. injection (426.5±51.0 vs. 131.2±57.6 arbitrary units for saline and LPS treated respectively) (n=4 animals per treatment). Statistical significance was determined using an unpaired Student's t-test; $p<0.05$.

FIGURE 5-3

The Effect of Cytokines Administered by i.c.v. Injection on CYP1A Activity in the Brain and Liver. Each drug treatment represents an individual experiment with the solid bars representing the control animals and the clear bars, treated animals (n=4 animals per treatment). **(A)** EROD activity determined in brain membrane fractions. The saline control for the TNF-α experiment = 0.24±0.02 pmol. resorufin/mg prot./min. In animals treated with a 2.5 ng dose of IL-1β the control value = 0.69±0.11 pmol resorufin/mg prot./min. The control activity for the IFN-γ =0.03±0.004 pmol. resorufin/mg prot./min. for the saline control. **(B)** EROD activity in liver microsomes. Hepatic EROD activity is 97.5±9.6 pmol. resorufin/mg prot./min. for TNF-α control, 84.9±6.4 pmol. resorufin/mg prot./min. for the IL-1β control, and 43.7±2.1 pmol. resorufin/mg prot./min. for the IFN-γ control. Note that the data for TNF-α has previously been reported (Renton and Nicholson, 2000). Statistical significance was determined using an unpaired Student's t-test; $p<0.05$.

*significantly different with respect to corresponding saline control.


A
Percent of Control Activity
140
120
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60
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TNF-alpha
IL-1beta
IFN-gamma
Drug Treatment
B
Percent of Control Activity
140
120
100
80
60
40
20
0
TNF-alpha
IL-1beta
IFN-gamma
Drug Treatment

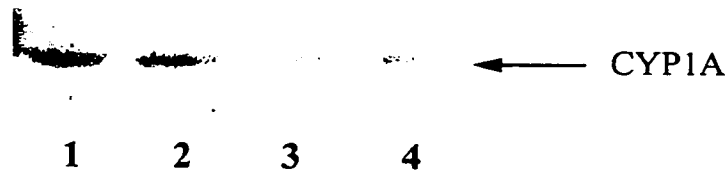
FIGURE 5-3

liver was seen when a dose of 50 ng IL-1β was injected into the lateral ventricle (data not shown). IFN-γ injected into the lateral ventricle resulted in an 88% decrease in brain EROD activity again with no effect in liver (Fig. 5-3A&B). CYP1A protein levels in brain appeared to be depressed by an i.c.v. injection of IFN-γ as measured by Western blotting (Fig. 5-4). In contrast to the results obtained with the other three cytokines, IL-1α and IL-6, which are also released in response to LPS, had no effect on either brain or liver EROD activity when administered into the lateral ventricle (Table 5-1).

The sensitivity of the liver to cytokines was demonstrated by administering cytokines by i.p. injection. Animals treated with either 0.4 μg TNF-α or 50 ng of IFN-γ by i.p. injection caused a 43% and 36% decrease in liver EROD respectively. The enzyme activity in the brains of these animals remained unchanged (Table 5-2). In contrast, IL-1β had no effect on EROD activity in either liver or brain at the doses examined (Table 5-2).

Hsp27 is expressed in the brain following LPS treatment. The occurrence of an immune/inflammatory response in the CNS during these experiments was demonstrated by examining brain sections for markers of inflammation. Robust staining for heat shock protein 27 (hsp27) was observed in the outer cortex, hippocampus, and surrounding the ventricles on both the ipsilateral and contralateral sides of LPS treated animals (Fig. 5-5C&D). In contrast, no expression of hsp27 was observed in saline treated animals (Fig. 5-5A&B). In a similar manner, animals treated with TNF-α i.c.v. had no detectable levels of hsp27 staining (Fig. 5-5E&F). Minimal patterns of staining were observed in

FIGURE 5-4



Effect of Cytokines Given by i.c.v. Injection on CYP1A Protein in the Brain. Representative Western blot for animals treated with an i.c.v. injection of IFN-γ. Each lane contains 37.5 μg of brain protein. Lanes 1&2 are saline treated animals, lanes 3&4 are animals that received 5 ng of IFN-γ. The relative densities are 1934 and 736 arbitrary units for saline and IFN-γ treated animals respectively (n=2 animals per treatment).

TABLE 5-1

Effects of acute phase cytokines given by i.c.v. injection on CYP1A activity in brain and liver.

Treatment	Tissue	Activity ± SEM
Saline i.c.v.	Brain	0.27±0.07 pmol. res./mg prot./min.
5 ng IL-1α i.c.v.		0.21±0.01 pmol. res./mg prot./min.
Saline i.c.v.	Liver	160.9±5.0 pmol. res./mg prot./min.
5 ng IL-1α i.c.v.		210.9±17.8 pmol. res./mg prot./min.
PBS i.c.v.	Brain	0.14±0.02 pmol. res./mg prot./min.
20 ng IL-6 i.c.v.		0.13±0.02 pmol. res./mg prot./min.
PBS i.c.v.	Liver	117.2±12.3 pmol. res./mg prot./min.
20 ng IL-6 i.c.v.		110.6±7.7 pmol. res./mg prot./min.

TABLE 5-2

Effects of cytokines given i.p. on levels of CYP1A activity in brain and liver.

Treatment	Tissue	Activity ± SEM
Saline i.p.[a]	Brain	0.39±0.06 pmol. res./mg prot./min.
0.4 μg TNF-α i.p.		0.30±0.03 pmol. res./mg prot./min.
Saline i.p. [a]	Liver	128.6±11.2 pmol. res./mg prot./min.
0.4 μg TNF-α i.p.		74.9±11.7 pmol. res./mg prot./min.[b]
Saline i.p.	Brain	0.42±0.03 pmol. res./mg prot./min.
0.1 μg IL-1β i.p.		0.40±0.06 pmol. res./mg prot./min.
Saline i.p.	Liver	158.9±21.4 pmol. res./mg prot./min.
0.1 μg IL-1β i.p.		162.3±18.9 pmol. res./mg prot./min.
Saline i.p.	Brain	0.27±0.03 pmol. res./mg prot./min.
50 ng IFN-γ		0.29±0.02 pmol. res./mg prot./min.
Saline i.p.	Liver	102.8±3.8 pmol. res./mg prot./min.
50 ng IFN-γ		65.5±7.4 pmol. res./mg prot./min.[b]

[a]TNF-α results taken from Renton and Nicholson, 2000.
[b]significant with respect to the saline control.

FIGURE 5-5

The Effect of LPS or Cytokines on Hsp27 Expression in the Brain. Coronal sections were stained with a primary antibody targeted against murine hsp25. Robust staining was seen in the hippocampus and surrounding the lateral ventricles of LPS treated animals (**C&D** respectively) whereas saline controls had virtually no staining in either area **(A&B)**. Animals treated with 5 ng of TNF-α i.c.v. had no hsp27 staining in the lateral ventricle nor in the hippocampus **(E&F)**. Scale bar = 200 μm.

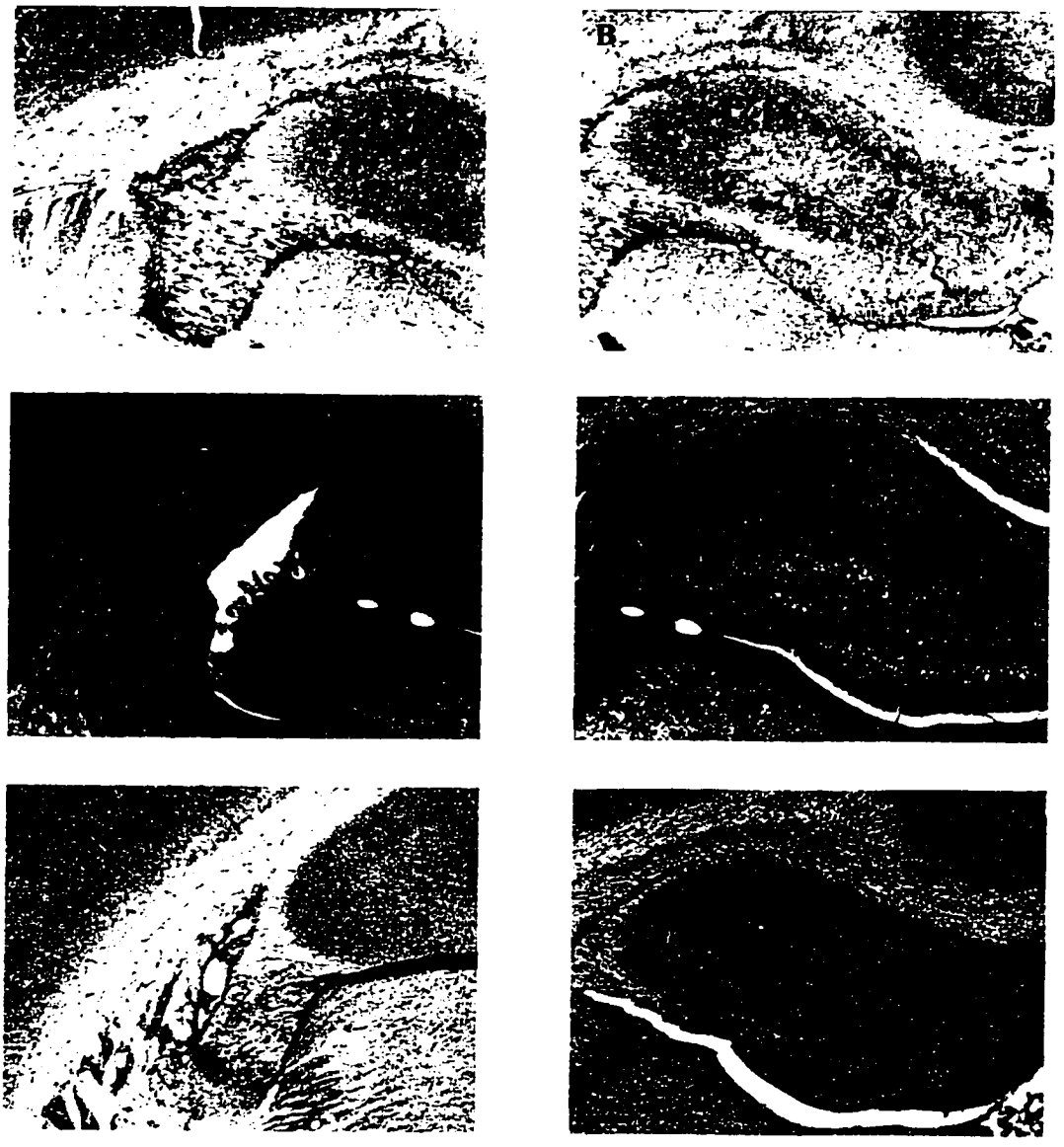


FIGURE 5-5

animals treated with i.c.v. injections of IL-1α, IL-1β, or IL-6 (data not shown). The negative controls (absence of the primary antibody) did not contain any staining.

The activation of microglia. Robust staining for microglia was observed throughout the thalamic area, adjacent to the ventricles, and in the hippocampus of LPS treated animals (Fig. 5-6C&D). In saline treated controls (Fig. 5-6A&B), some staining could be observed around the ventricles and in the hippocampus, but to a much lesser extent than observed with LPS treated animals. In response to an i.c.v. injection of TNF-α, microglia were observed in the thalamic area, surrounding the ventricles, and in the hippocampus although to a much lesser extent than seen in LPS treated animals (Fig. 5-6E&F). In contrast, animals treated with IL-1α, IL-1β, or IL-6 demonstrated little or no staining (data not shown). The negative control did not contain any detectable staining.

Peripheral cytokine levels following i.c.v. LPS administration. Cytokine levels in serum at various times following the i.c.v. administration of saline, LPS, TNF-α, and IL-1β are shown in figure 5-7. The levels of TNF-α, IL-1β, IFN-γ, and IL-6 were minimal over a 24-hour time period in animals receiving an i.c.v. injection of saline. Substantial levels of all four cytokines occurred in blood in response to an i.c.v. injection of LPS. The profiles of cytokine release generally showed a peak in release between 2 and 4 hours with levels dropping off to near undetectable levels by 12 hours. The levels of these four cytokines in animals injected i.c.v. with either TNF-α or IL-1β was similar to that observed in saline treated animals.

FIGURE 5-6

Microglial Expression in the Brain. A 24-hour exposure to LPS or cytokines led to an increase in microglial expression throughout the brain. Saline controls showed some staining surrounding ventricles and through the hippocampus (**A&B** respectively). In contrast, LPS treated animals demonstrated a high level of microglial activity throughout the hippocampus, and around the ventricles (**C&D** respectively). TNF-α showed a modest amount of microglial staining in the hippocampus and surrounding the ventricles **(E&F)**. Scale bar = 200 μm.

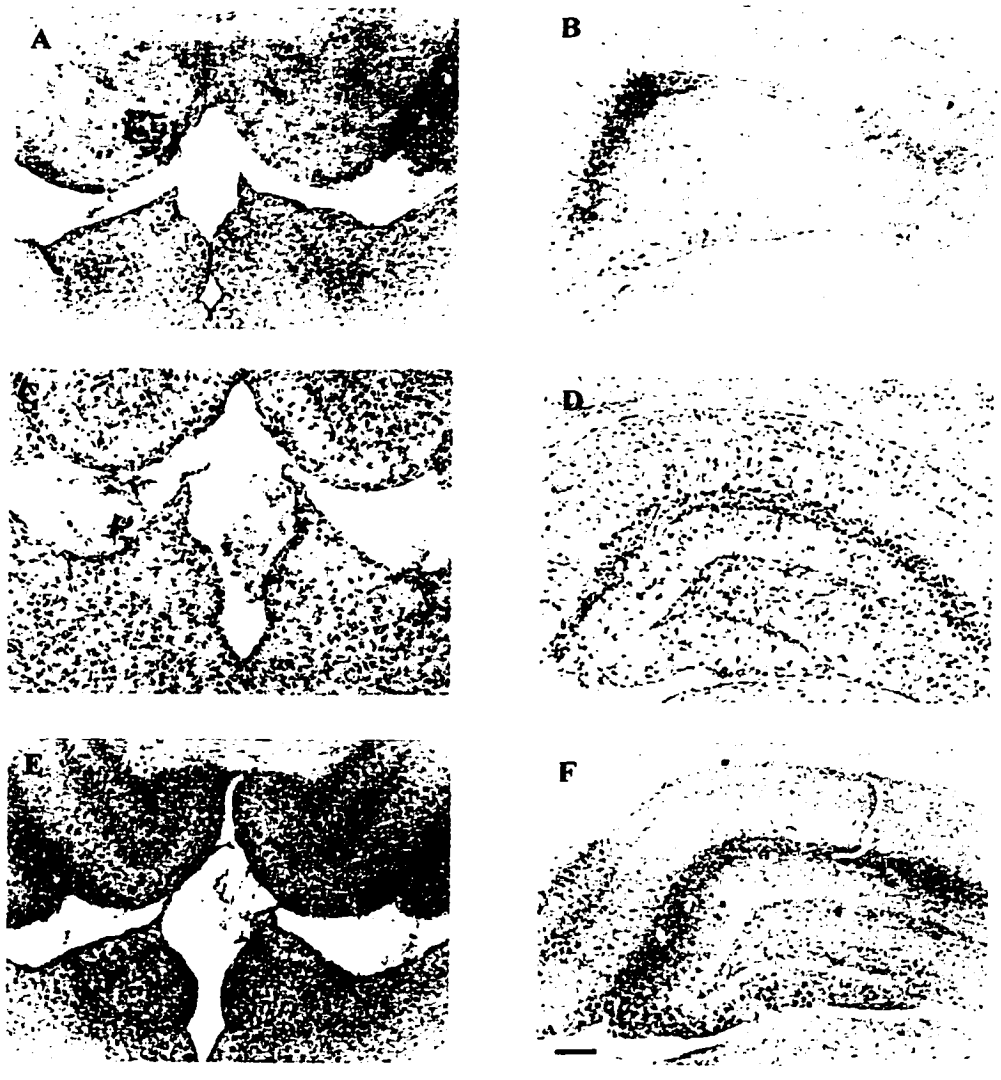
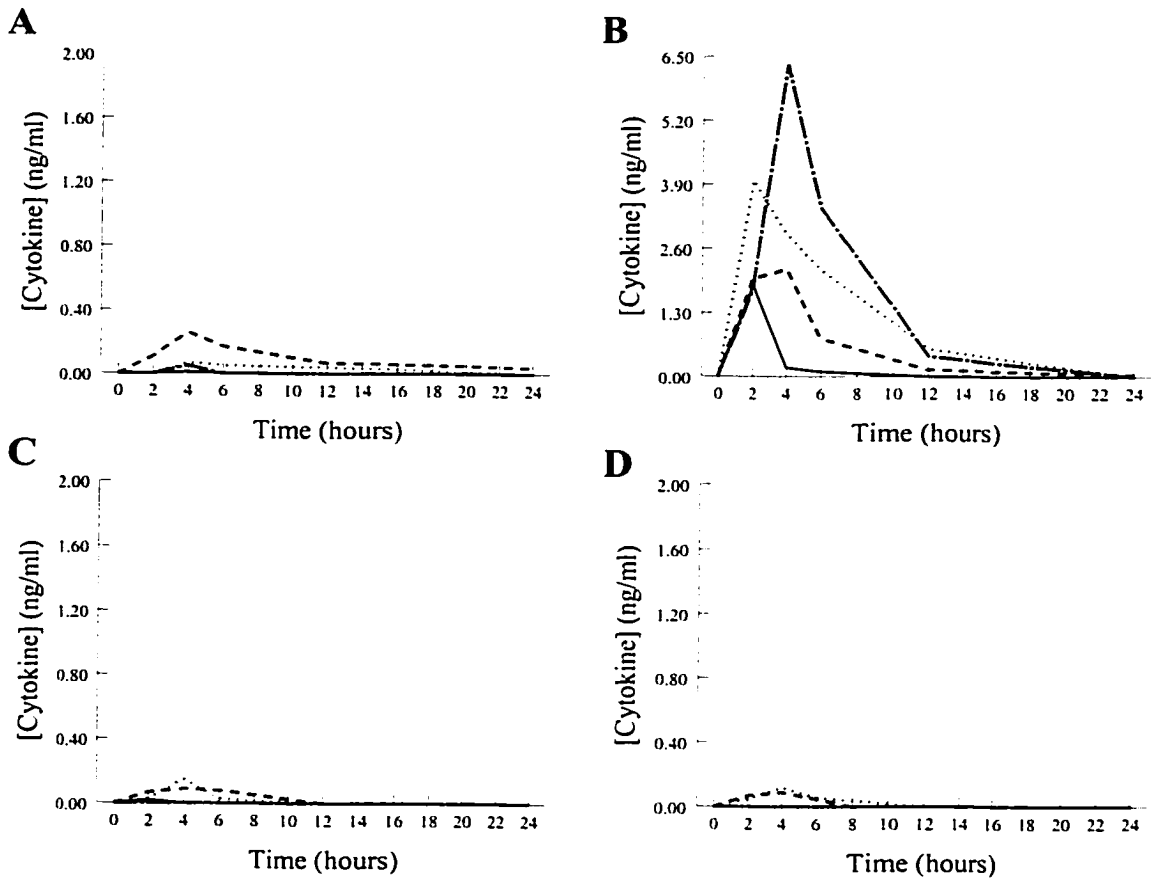


FIGURE 5-6

FIGURE 5-7



Appearance of Cytokines in Serum Following Drug Treatment. Time course for the appearance of TNF-α, IL-1β, IFN-γ, and IL-6 in serum samples of animals treated with an i.c.v. injection of **(A)** saline, **(B)** 25 μg LPS, **(C)** 5 ng TNF-α, or **(D)** 2.5 ng IL-1β. Each time period represents the mean value from two animals.

5.4 DISCUSSION

Systemic administration of LPS differentially depresses the activity of a variety of hepatic cytochrome P450 isoforms (Franklin and Finkle, 1985; Ghezzi et al., 1986a; Ghezzi et al., 1986b; Poüs et al., 1990; Stanley et al., 1991; Craig et al., 1993; Ferrari et al., 1993; Morgan et al., 1994). Ghezzi et al. (1986c), used an LPS-resistant mouse strain (C3H/HeJ) to show that serum from LPS responsive animals depressed hepatic ethoxycoumarin deethylase activity in these C3H/HeJ animals. This provided strong evidence that a serum factor was mediating the effects of LPS on hepatic cytochrome P450 activity. In addition, they showed that injection of IL-1 could depress hepatic cytochrome P450 activity in these LPS-resistant mice. Subsequently, others have demonstrated that systemic administration of cytokines such as TNF-α, IL-1β, IL-1α, IFNs, and to some extent IL-6 could differentially depress levels of hepatic cytochrome P450 activity (Franklin and Finkle, 1985; Ghezzi et al., 1986c; Poüs et al., 1990; Craig et al., 1993; Ferrari et al., 1993; Morgan et al., 1994). Since one of the primary actions of LPS *in vivo* is the release of cytokines via the activation of immunocompetent cells, these effects are not surprising.

Over the 24 hour study period used in the present experiments, LPS injected into the lateral ventricle of the brain significantly depressed CYP1A activity in the CNS. CYP1A protein levels in the brain were decreased by the i.c.v. injection of LPS implicating a pre-translational mechanism of enzymatic inhibition or possibly increased protein degradation. These observations are in agreement with earlier reports from this lab where a decrease in CYP1A staining was seen in brain sections of animals treated with an i.c.v. injection of LPS (Renton et al., 1999). The experiments described here in

part attempted to determine whether cytokines could mediate this LPS induced decrease in CYP1A activity within the CNS. When injected into the lateral ventricle, LPS activates immunocompetent cells within the brain parenchyma to release pro-inflammatory cytokines (Fabry et al., 1993). LPS induces the cytokine cascade by promoting the synthesis and release of TNF-α, which activates the synthesis of IL-1 by binding to its cellular receptor. IL-1 in turn binds to its cell surface receptor and stimulates the release of IL-6 (Dantzer et al., 1999). These cytokines have a diverse number of actions *in vivo*, including the activation of immune cells, stimulation of the hypothalamic-pituitary-adrenal axis, induction of fever, and sickness behaviours (Di Santo et al., 1999). To investigate the possibility that cytokines are responsible for mediating the effects of centrally administered LPS on CYP1A activity, recombinant cytokines were administered by i.c.v. injection and the effects on CYP1A assessed. The pro-inflammatory cytokines, TNF-α and IL-1β both significantly decreased CYP1A activity in the brain indicating that they are likely candidates for mediating the effects of LPS. This was not uniformly observed with all acute phase or pro-inflammatory cytokines as neither IL-1α nor IL-6 had any effect on brain CYP1A activity when administered by i.c.v. injection. Previous work using *in vitro* models has shown that IL-6 is capable of depressing the activity of various cytochrome P450 isoforms in hepatocytes (Williams et al., 1991; Fukuda et al., 1992; Fukuda and Sassa, 1994). However, when administered *in vivo*, IL-6 has a variety of different effects depending on the isoform examined (Morgan, 1991; Morgan et al., 1994). In our experiments, IL-6 did not affect brain CYP1A activity when injected i.c.v. indicating that this cytokine is not directly involved in the effects of LPS observed in this model. Somewhat surprising was the

observation that IL-1α did not have any effect on CNS CYP1A activity as the biological activities of both IL-1 isoforms are mediated through the same receptor and IL-1β significantly depressed CYP1A activity (Anforth et al., 1998). However, it has been previously reported that the relative potencies of these two isoforms of IL-1 are not the same but depend on various factors such as the endpoint measured.

The anti-viral cytokine, IFN-γ is released in response to LPS and has effects on hepatic cytochrome P450 when administered systemically (Ho, 1964; Renton and Mannering, 1976a). Injection of this cytokine into the lateral ventricle also resulted in a substantial loss of CYP1A activity in the brain and a reduction in protein expression. IFN-γ is one of the primary mediators released in response to viral infections, and thus could alter local drug handling during a CNS infection.

A more surprising result from this work and others (Shimamoto et al., 1998; Renton and Nicholson, 2000) is that LPS given i.c.v. can depress levels of CYP1A2 activity in peripheral organs such as the liver. Examination of protein levels revealed that the depression of CYP1A2 activity seen in liver resulted from a down-regulation of CYP1A2 protein. Similar results obtained by Shimamoto et al. found that hepatic CYP1A2 activity and protein were decreased following LPS administration in the brain (Shimamoto et al., 1998). There are several possible mechanisms by which centrally administered LPS can depress CYP1A2 activity in liver; centrally produced cytokines, activation of the HPA axis, sympathetic nerve stimulation, or peripherally produced cytokines. It was postulated that the effects of centrally administered LPS on hepatic CYP1A2 activity might result from the local induction of cytokine synthesis and release. However, we have now shown that when hepatic CYP1A2 activity was assessed in

animals that received centrally administered cytokines, no alterations in enzymatic activity were seen for any of the cytokines examined at the dosages used. It was initially thought that higher doses of cytokines may be needed to mimic the effects of LPS on CYP1A2 activity in the liver. However, when a high dose of IL-1β (50 ng) was injected into the lateral ventricle, no effect was seen on CYP1A2 activity in the liver. The inability of these pro-inflammatory cytokines to have an effect on hepatic CYP1A2 activity when administered into the CNS suggests that local production of cytokines within the brain do not trigger a signaling pathway involved in the down-regulation of CYP1A2 activity as seen with LPS. It also could be hypothesized that LPS alters the BBB allowing centrally produced cytokines to enter the peripheral circulation and induce further activation of the immune response. This also does not seem to account for the results obtained here as the breakdown of the BBB is normally not compromised for approximately 48 hours following immune challenge and these experiments were conducted 24 hours after LPS administration (Perry et al., 1997).

Shimamoto et al. demonstrated that activation of the HPA axis does not mediate the effects of an i.c.v. injection of LPS on cytochrome P450 in the liver as adrenalectomy failed to prevent this effect. In fact, they found that adrenalectomy depressed levels of cytochrome P450 even further than seen with sham operated animals which were given LPS by i.c.v. injection (Shimamoto et al., 1999). This led them to suggest that it was perhaps peripherally released cytokines that may be involved in this effect. It has also been proposed that activation of the sympathetic nervous system may mediate the effects of centrally administered LPS on cytochrome P450 activity in liver. However, experiments by Shimamoto et al. (1999) showed an independence of this effect from

sympathetic stimulation as surgical or chemical sympathetecomized animals responded to centrally administered LPS by decreasing hepatic cytochrome P450 activity in a similar manner to controls. In addition, our laboratory has shown that the loss of hepatic CYP1A2 activity resulting from an i.c.v. injection of LPS is unaffected by β-receptor blockade with propranolol (Renton and Nicholson, 2000). Work by De Luigi et al. (1998) suggests that the sympathetic nervous system tonically inhibits the peripheral release of cytokines as sympathectomy increased the production of cytokines in response to centrally administered LPS. This increase in peripheral cytokine production following sympathectomy may account for the inability to prevent the decrease in CYP1A2 activity in the liver. These reports along with the results presented here support the idea that peripherally derived cytokines might be crucial for mediating the effects of an i.c.v. injection of LPS on CYP1A2 activity in the liver.

High concentrations of the pro-inflammatory cytokines TNF-α, IL-1β, IFN-γ, and IL-6 were observed in peripheral blood in the first few hours after LPS administration into the lateral ventricle. We demonstrated that peripherally derived cytokines are capable of altering hepatic CYP1A2 activity. This was done by showing that exogenously administered cytokines, calculated to yield similar doses to those measured in blood samples from LPS (i.c.v.) treated animals (Poüs et al., 1990), depress hepatic CYP1A2 activity when given by i.p. injection. This confirms observations first made by others (Ghezzi et al., 1986b; Ghezzi et al., 1986c; Stanley et al., 1988; Ferrari et al., 1993). From these results it appears that the induction of peripheral cytokine release is consistent with the idea that they may mediate the effects of centrally administered LPS on hepatic CYP1A activity.

The induction of an immune response has classically been considered a requirement for the LPS induced decrease of cytochrome P450 activity (Ghezzi et al., 1986c; Morgan, 1989). Immunohistochemical studies reported here demonstrate that i.c.v. LPS induces a robust expression of hsp27 in the brain, a classic marker of stress (Hauser et al., 1996). In addition, LPS induced the activation of numerous microglia in the hippocampus, ventricles, and thalamus. These results did not seem to be mediated through the production of cytokines TNF-α, IL-1β, IL-1α, and IL-6 as the injection of these cytokines did not induce the expression of either hsp27 or activate microglia. Previous reports concerning the effects of cytokines on hsp27 indicated that they do not induce levels of hsp27 but alter its phosphorylation state, which is consistent with the results described here (Satoh and Kim, 1995). It is possible that the global immune response mediated by LPS may be necessary for the decrease in CYP1A2 activity in liver. This is supported by results from these experiments as i.c.v. injection of cytokines could not mimic the immune activation in brain as assessed by immunohistochemistry, nor could they decrease CYP1A2 activity in the liver. In addition to cytokines, LPS is known to activate other immunological mediators such as the arachidonic acid metabolites and inducible nitric oxide synthase (iNOS) both of which could potentially participate in mediating this brain to liver response (Boje and Arora, 1992; Sehic et al., 1996). Although the induction of cytokines in the periphery seems to play an important role in this effect, it should be recognized that they are not the only mediators.

In conclusion, we have demonstrated that cytokines participate in the down-regulation of CYP1A activity in the brain in response to inflammation within the CNS. In contrast, the concomitant modulation of cytochrome P450 in the liver during a CNS

inflammatory response is not mediated by a pathway involving centrally produced cytokines. However, the subsequent induction of cytokines outside the brain likely participates in the depression of this enzyme in peripheral organs. It is most likely that the effects on CYP1A activity in liver are not mediated by one system alone but result from a complex interplay between the HPA axis, nervous system, and generation of peripheral immune mediators.

CHAPTER 6

MODULATION OF CYTOCHROME P450 IN LIVER DURING CENTRAL INFLAMMATION INDUCED BY LPS

6.0 ABSTRACT

Infection and inflammation within the CNS results in the modulation of cytochrome P450 enzymes in the brain and in the liver. These alterations in enzymatic activity can have serious complications including drug toxicity and/or disruption of normal physiological functions. In patients with neurodegenerative disorders that involve a chronic inflammatory component such as MS, Parkinson's and Alzheimer's disease, this disregulation of hepatic cytochrome P450 activity could have serious consequences.

In this study we investigated the potential role of cytokines in mediating the decrease in total hepatic cytochrome P450, CYP2B1 and 2E1 activity during a central inflammatory response induced by i.c.v. injection of the bacterial endotoxin LPS. We report that cytokines produced in brain do not seem to be involved in the alteration of hepatic cytochrome P450 activity whereas a potential role for the peripheral production of the acute phase cytokine TNF-α following i.c.v. injection of LPS is proposed.

6.1 INTRODUCTION

In response to an immune or inflammatory condition, cytochrome P450 enzymatic activity is depressed in the liver (reviewed by Morgan (1997)). This has serious implications for drug effectiveness and/or toxicity, in particular for pro-drugs and those agents that have a narrow therapeutic index (Chang et al., 1978; Morgan, 1997). Some evidence has been presented demonstrating a down-regulation of cytochrome P450 isoforms in the liver during an inflammatory response localized to the brain (Shimamoto et al., 1998; Renton and Nicholson, 2000). Peripherally derived cytokines released in response to this CNS inflammatory reaction are thought to partially mediate the effects on CYP1A2 activity in liver (Nicholson and Renton, 2001c). This type of regulation of cytochrome P450 activity in the liver could have serious consequences for patients with acute central infections and/or inflammatory responses following brain tissue injury and also for patients with neurodegenerative disorders such as MS, Parkinson's and Alzheimer's disease which are thought to involve a chronic inflammatory component (Hauss-Wegrzyniak et al., 1998).

In this paper we examine the role of centrally administered cytokines on total hepatic cytochrome P450 content, CYP2B1 and 2E1 activity in liver and compare them to the effects of cytokines administered peripherally. We demonstrate that centrally administered cytokines do not affect any of these measures when injected by the i.c.v. route. In contrast, when injected i.p., cytokines differentially affect cytochrome P450 activity and total content.

6.2 MATERIALS AND METHODS

Tissue preparations and drug treatments were carried out as described in chapter 2.

6.3 RESULTS

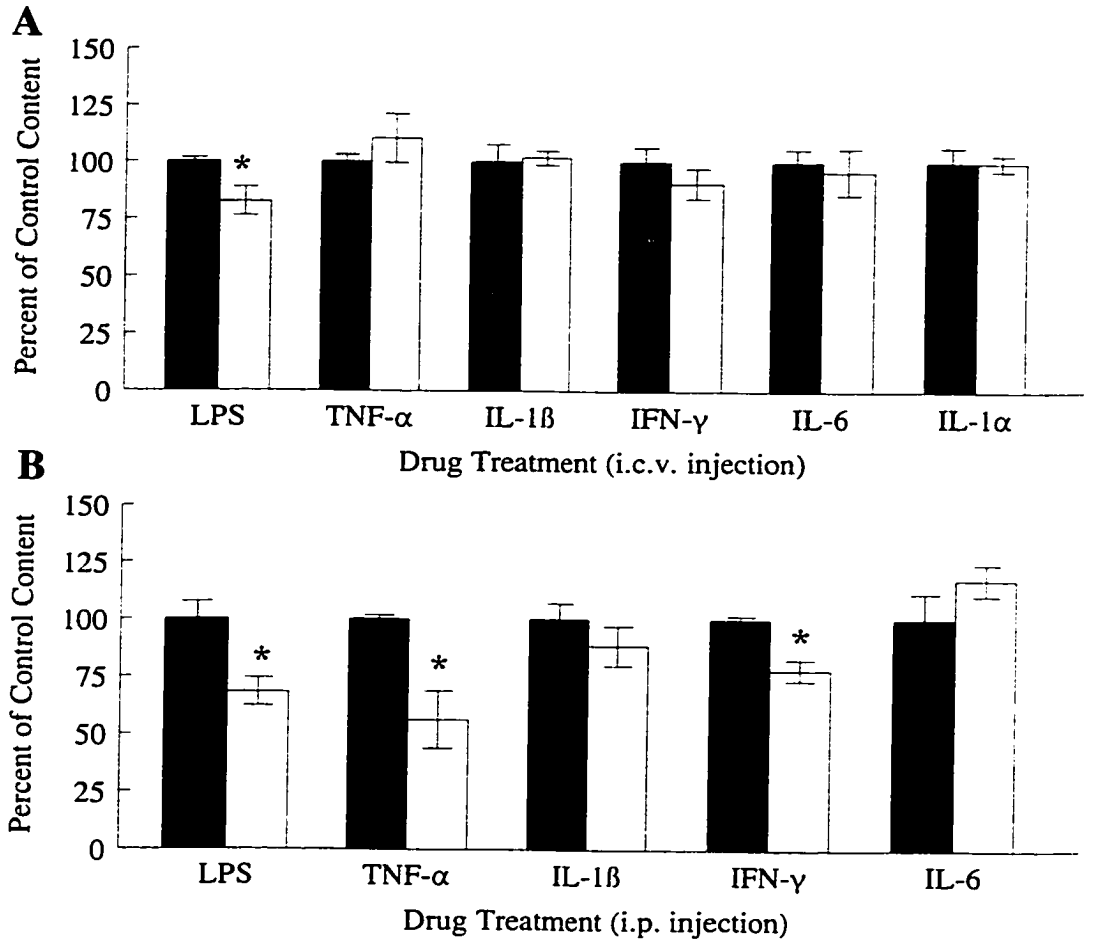
LPS and cytokines evoke a loss of hepatic cytochrome P450 content. Total hepatic content of cytochrome P450 was determined in microsomes obtained from animals 24 hours after receiving an i.c.v. injection of LPS or cytokines (Fig. 6-1A). LPS significantly decreased total P450 content by 17% from control levels. The acute phase cytokines, TNF-α (5 ng), IL-1β (2.5 ng), IFN-γ (5 ng), IL-6 (20 ng), or IL-1α (5 ng), did not alter total hepatic cytochrome P450 content when injected into the lateral ventricle of the rat. A high dose IL-1β (50 ng) was also assessed and no alteration in cytochrome P450 content was seen suggesting that the lack of effect was not due to the dose chosen (data not shown).

To confirm that hepatic cytochrome P450 content is in fact sensitive to cytokines, these same acute phase cytokines were given via the i.p. route. Total hepatic cytochrome P450 content was decreased by several of the cytokines (Fig. 6-1B). Administration of 0.5 mg/kg LPS was used as a positive control and was found to decrease total hepatic cytochrome P450 content by 32% from control 24 hours after injection. Administration of 0.4 μg of TNF-α decreased levels of cytochrome P450 content by 44% and in a similar manner, administration of 50 ng of IFN-γ suppressed hepatic cytochrome P450 content by 22% from a control. Neither IL-1β (0.1 μg) nor IL-6 (0.5 μg) could affect a significant alteration in total hepatic content of cytochrome P450.

FIGURE 6-1

Hepatic Cytochrome P450 Content is Differentially Affected by LPS and Cytokines. Animals were given an **(A)** i.c.v. or **(B)** i.p. injection of either LPS or cytokines and total hepatic content of cytochrome P450 was determined 24 hours later. The solid bars represent the saline controls from individual experiments and the hollow bars the treated animals (n=4 animals per treatment). The saline controls for the i.c.v. experiments are 0.63±0.01, 0.66±0.04, 0.65±0.05, 0.76±0.05, 0.68±0.04, and 0.88±0.06 nmoles P450/mg protein for LPS (25 μg), TNF-α (5 ng), IL-1β (2.5 ng), IFN-γ (5 ng), IL-6 (20 ng), and IL-1α (5 ng) respectively. The saline controls for the i.p. injections are 0.66±0.05, 0.64±0.01, 0.70±0.05, 0.64±0.01, and 0.40±0.05 nmoles P450/mg protein for LPS (0.5 mg/kg), TNF-α (0.4 μg), IL-1β (0.1 μg), IFN-γ (50 ng), and IL-6 (0.5 μg) respectively. Statistics were performed on raw data from individual experiments using an unpaired Student's t-test, $p<0.05$.

*significant with respect to saline control.


A
Percent of Control Content
150
125
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75
50
25
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*
LPS
TNF-α
IL-1ß
IFN-γ
IL-6
IL-1α
Drug Treatment (i.c.v. injection)
B
Percent of Control Content
150
125
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75
50
25
0
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*
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LPS
TNF-α
IL-1ß
IFN-γ
IL-6
Drug Treatment (i.p. injection)

FIGURE 6-1

Differential effects of LPS and cytokines on hepatic CYP2B1 activity depending on route of administration. When drugs are administered through the i.c.v. route, hepatic CYP2B1 activity, as assessed by the PROD assay, is sensitive to regulation by LPS and IL-1β (Fig. 6-2A). Levels of CYP2B1 activity were suppressed by 51% from controls in response to LPS. A 65% decrease in CYP2B1 protein levels following i.c.v. injection of LPS mirrored the effects seen on activity (Fig. 6-3A). Surprisingly, IL-1β (2.5 ng) significantly increased PROD activity by 42% over control. In comparison, none of the other acute phase cytokines had any significant effect on CYP2B1 activity.

In contrast to the effects of cytokines when given by i.c.v. injection, administration via the i.p. route showed that CYP2B1 activity is decreased by 29% in response to 0.4 μg of TNF-α (Fig. 6-2B). None of the other acute phase cytokines had a significant effect on CYP2B1 activity at the doses examined. The positive control LPS, suppressed PROD activity by 50%, however, protein levels were not found to be significantly different (Fig. 6-3B).

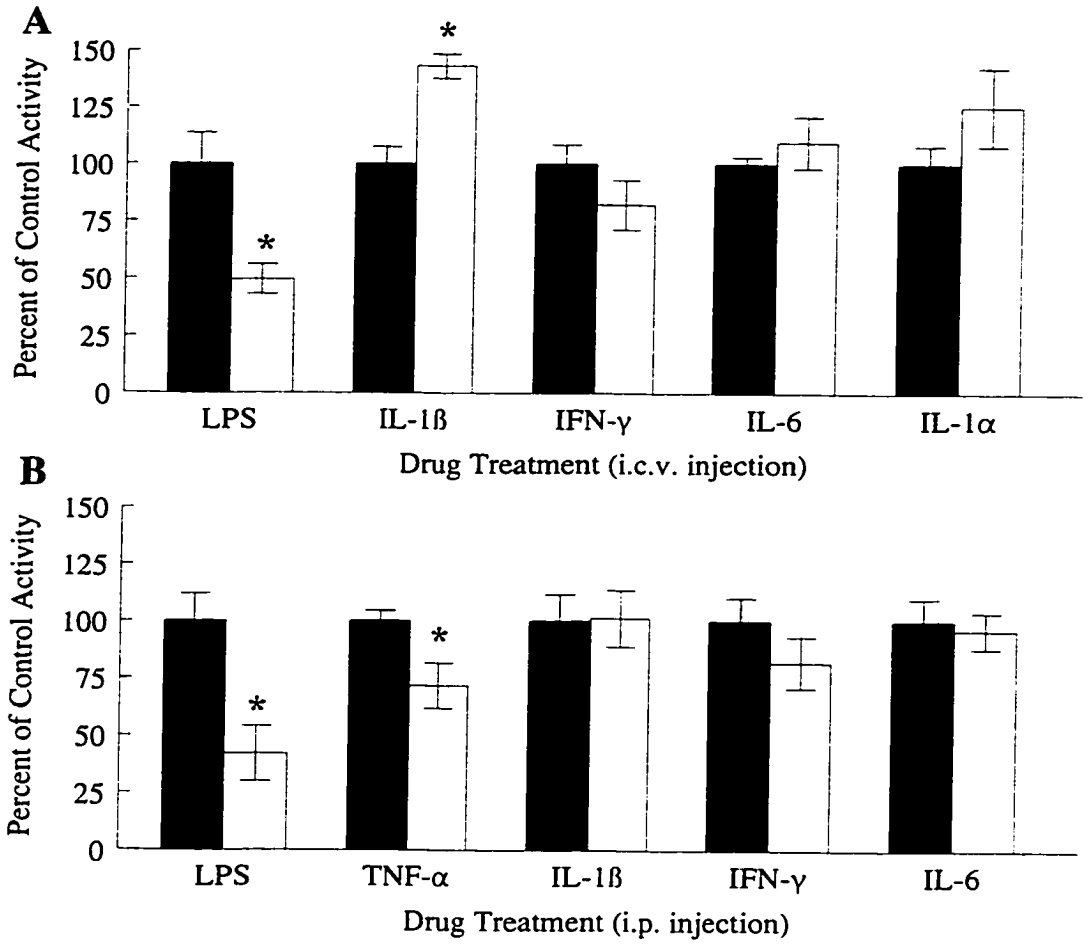
Differential effects of cytokines on CYP2E1 activity in the liver. When injected via the i.c.v. route only LPS had an effect on CYP2E1 activity in liver (Fig. 6-4A). 25 μg of LPS decreased hepatic CYP2E1 activity by 55% 24 hours after administration, however CYP2E1 protein levels of treated animals were not significantly different from control levels (Fig. 6-5A). In comparison, none of the acute phase cytokines given by i.c.v. injection altered hepatic CYP2E1 activity.

Injection of 0.4 μg of TNF-α by i.p. injection resulted in a 31% decrease in

FIGURE 6-2

LPS and Cytokines have Differential Effects on Hepatic CYP2B1 Activity. Animals were injected **(A)** i.c.v. or **(B)** i.p. with either LPS or cytokines and CYP2B1 activity was assessed by measuring PROD activity 24 hours later. The solid bars represent saline controls from individual experiments and the hollow bars the treated animals from those same experiments (n=4 animals per treatment). The saline controls for the i.c.v. experiments are 22.0±3.0, 13.2±1.0, 23.1±2.0, 12.4±0.4, and 19.6±1.6 pmol. resorufin/mg protein/min. for LPS (25 μg), IL-1β (2.5 ng), IFN-γ (5 ng), IL-6 (20 ng), and IL-1α (5 ng) respectively. The saline controls for the i.p. injections are 10.0±1.2, 22.3±1.0, 15.8±1.9, 11.6±1.2, and 10.0±1.0 pmol. resorufin/mg protein/min. for LPS (0.5 mg/kg), TNF-α (0.4 μg), IL-1β (0.1 μg), IFN-γ (50 ng), and IL-6 (0.5 μg) respectively. Statistics were performed on raw data using an unpaired Student's t-test, $p<0.05$.

*significant with respect to saline control.


A
Percent of Control Activity
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125
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75
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LPS
IL-1ß
IFN-γ
IL-6
IL-1α
Drug Treatment (i.c.v. injection)
B
Percent of Control Activity
150
125
100
75
50
25
0
*
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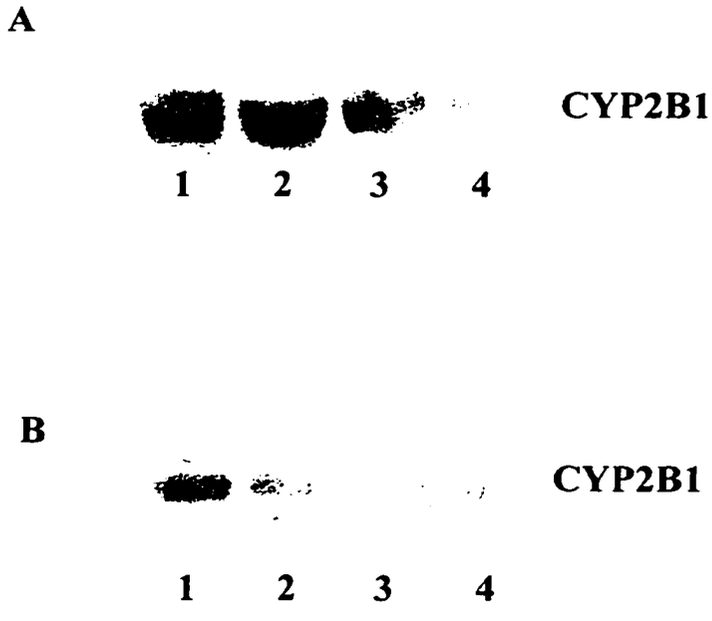
LPS
TNF-α
IL-1ß
IFN-γ
IL-6
Drug Treatment (i.p. injection)

FIGURE 6-2

FIGURE 6-3



Representative Western Blots for Hepatic CYP2B1 Protein in Treated Animals. 25 μg of hepatic microsomal protein was separated on a 10% ready gel under non-reducing conditions. Blots were probed for CYP2B1 immunoreactive protein and in each case lanes 1&2 represent saline controls and lanes 3&4 treated animals. **(A)** CYP2B1 protein densities were measured to be 753 vs. 266 arbitrary units for the saline controls and LPS treated animals respectively (n=2 animals per treatment). **(B)** Animals that received 0.5 mg/kg LPS (i.p.) had a protein density of 428±182 vs. 99±25 arbitrary units for saline controls (n=4 animals per treatment).

FIGURE 6-4

Differential Effects of LPS and Cytokines on Hepatic CYP2E1 Activity. The 6-hydroxylation of CZX was measured in liver microsomes 24 hours after an **(A)** i.c.v. or **(B)** i.p. injection of LPS or cytokines as an indicator of CYP2E1 activity. The solid bars represent saline controls from individual experiments and the hollow bars the treated animals from those same experiments (n=4 animals per treatment). The saline controls for the i.c.v. experiments are 82.2±6.9, 67.7±3.4, 75.3±4.0, 60.0±1.8, and 56.9±3.7 ng 6-OH CZX/mg protein/min. for LPS (25 μg), IL-1β (2.5 ng), IFN-γ (5 ng), IL-6 (20 ng), and IL-1α (5 ng) respectively. The saline controls for the i.p. injections are 84.5±6.1, 53.1±1.0, 116.0±10.8, 59.0±4.9, and 67.6±10.2 ng 6-OH CZX/mg protein/min. for LPS (0.5 mg/kg), TNF-α (0.4 μg), IL-1β (0.1 μg), IFN-γ (50 ng), and IL-6 (0.5 μg) respectively. Statistics were performed on raw data from individual experiments using an unpaired Student's t-test, $p<0.05$.

*significant with respect to saline control.

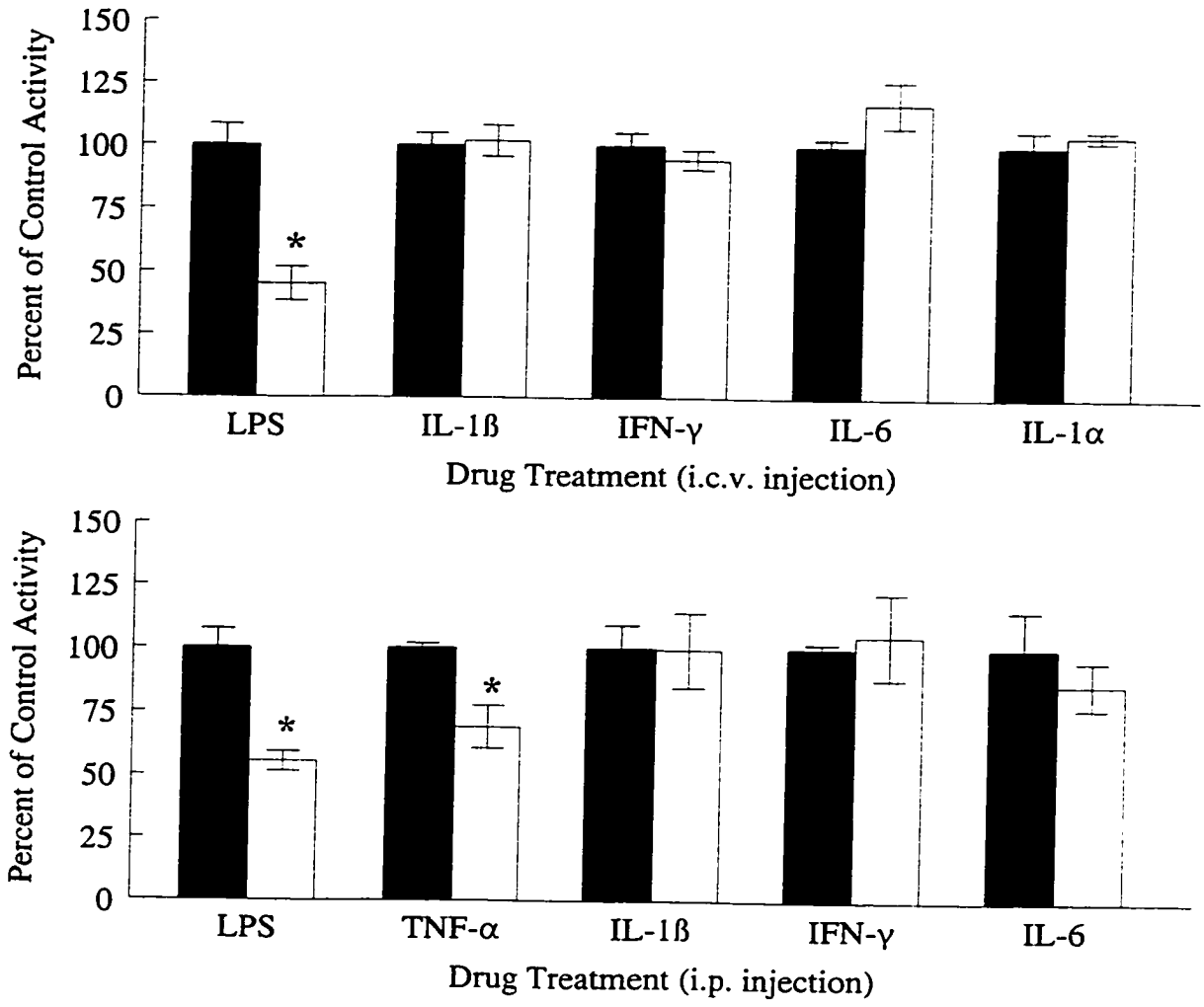

Percent of Control Activity
150
125
100
75
50
25
0
*
LPS
IL-1ß
IFN-γ
IL-6
IL-1α
Drug Treatment (i.c.v. injection)
Percent of Control Activity
150
125
100
75
50
25
0
*
*
LPS
TNF-α
IL-1ß
IFN-γ
IL-6
Drug Treatment (i.p. injection)

FIGURE 6-4

FIGURE 6-5

Representative Western Blots for CYP2E1 Protein in Treated Animals. 25 μg of hepatic microsomal protein was separated on a 10% ready gel under non-reducing conditions. Blots were probed for CYP2E1 immunoreactive protein and in each case lanes 1&2 represent saline controls and lanes 3&4 treated animals. **(A)** Animals that received 25 μg of LPS (i.c.v) had a protein density of 1455±169 vs. 1646±147 arbitrary units for the saline controls (n=4 animals per treatment). **(B)** Animals that received 0.4 μg TNF-α (i.p.) had a protein density of 2881 vs. 3845 for the saline controls (n=2 animals per treatment). **(C)** Animals that received 0.5 mg/kg LPS (i.p.) had a protein density of 1076±35 vs. 962±82 arbitrary units for saline controls (n=4 animals per treatment).

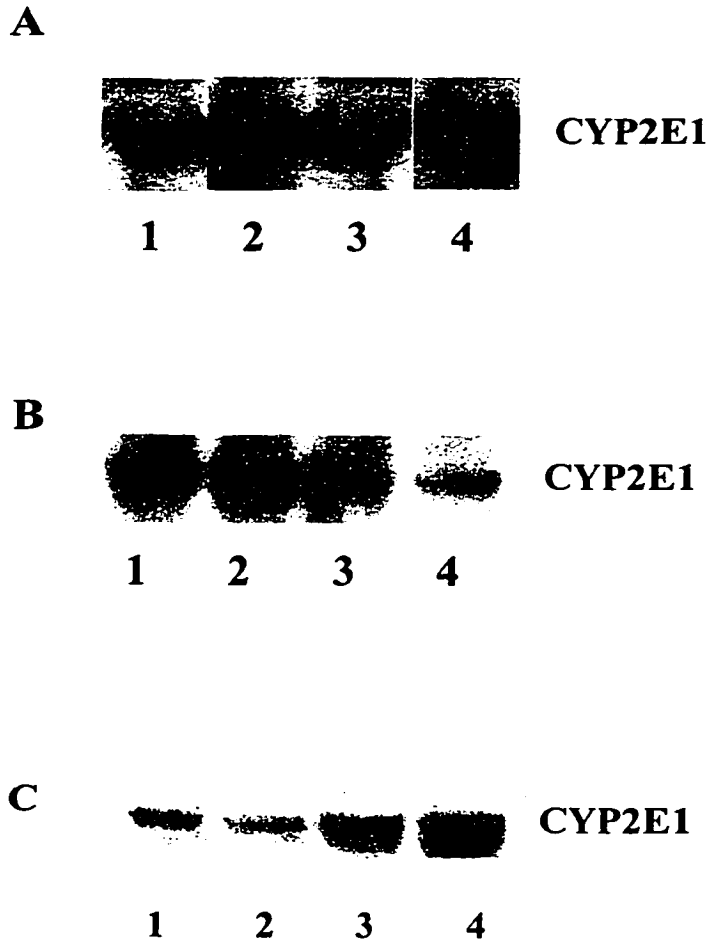


FIGURE 6-5

hepatic CYP2E1 activity (Fig. 6-4B). CYP2E1 protein levels were decreased by approx. 25% in a representative sample suggesting a down-regulation of the enzyme in response to this cytokine, however protein levels were not significantly different from control (Fig. 6-5B). None of the other acute phase cytokines had any significant effect on hepatic CYP2E1 activity. I.p. injection of LPS significantly decreased CYP2E1 activity by 45% but had no effect on CYP2E1 protein levels as judged by Western blotting (Fig. 6-5C).

6.4 DISCUSSION

The effects of infection and/or inflammation on cytochrome P450 activity can result in the aberrant handling of drugs and interfere with the endogenous functions of these enzymes. The effects of systemic infections are well known both in the clinical setting and in various animal models of experimentally induced infection or inflammation (Chang et al., 1978; Moore et al., 1983; Shedlofsky et al., 1987; Moochhala, 1991; Shedlofsky et al., 1994; Shedlofsky et al., 1997). A similar phenomenon has been shown to occur when utilizing an i.c.v. injection of LPS as a model of localized inflammation within the brain (Shimamoto et al., 1998; Renton and Nicholson, 2000). Using this model, the mechanism by which cytochrome P450 enzymes in the liver are modulated during a central inflammatory process was investigated and reported to be independent of the HPA axis and sympathetic nerve stimulation (Shimamoto et al., 1999; Renton and Nicholson, 2000). However, a role for peripherally derived cytokines has been reported for the modulation of the CYP1A2 isoform in liver (Nicholson and Renton, 2001c). In this section we investigated the ability of cytokines, administered by i.c.v. injection, to modulate cytochrome P450 content and activity in liver.

I.c.v. injection of 25 μg of LPS significantly suppressed hepatic cytochrome P450 content by 17%. Administration of LPS into the brain stimulates cytokine release within the brain parenchyma in a spatial-temporal pattern (Stern et al., 2000). To assess whether these centrally produced acute phase cytokines play a role in this effect TNF-α, IL-1α, IL-1β, IL-6, and IFN-γ were injected into the lateral ventricle of the rat to mimic the effects of locally produced cytokines in the brain parenchyma. Total hepatic cytochrome P450 was assessed in these animals and results showed that none of the acute phase cytokines altered this expression when injected by the i.c.v. route. A high dose of IL-1β (50 ng) was used to assess whether this was a result of inadequate cytokine concentration. When injected i.c.v., this dose of IL-1β was found to have no effect on cytochrome P450 content in liver indicating that the lack of effect by cytokines is probably not due to the concentrations used. The lack of effect on hepatic cytochrome P450 content suggests that centrally administered cytokines, given in isolation, are not a sufficient stimulus to induce a response that results in modulation of cytochrome P450 content in liver. Since LPS is capable of releasing several cytokines in addition to other immune mediators such as NO and PGE_2, it is perhaps this combined effect that results in the decreased hepatic cytochrome P450 content (Minghetti and Levi, 1998).

LPS induces the release of acute phase cytokines in the periphery when administered into the brain (Gottschall et al., 1992; Terrazzino et al., 1997; De Luigi et al., 1998; Borovikova et al., 2000; Nicholson and Renton, 2001c). It is therefore possible that peripherally derived cytokines alter the levels of hepatic cytochrome P450 content. To confirm that cytokines released in the periphery can modulate levels of cytochrome P450, we injected animals (i.p.) with several different acute phase cytokines and

measured the effects on cytochrome P450 activity. Both TNF-α and IFN-γ significantly depressed total hepatic P450 content confirming that hepatic cytochrome P450 is sensitive to regulation by cytokines but not when the cytokines are administered into the brain. This discrepancy most likely results from the inability of centrally administered cytokines to induce peripheral cytokine release, an effect we demonstrated in previous experiments (Nicholson and Renton, 2001c).

The modulation of specific cytochrome P450 isoforms showed similar trends to that observed with cytochrome P450 content as injection of 25 μg of LPS resulted in a 51% decrease in hepatic CYP2B1 activity. These results are in good agreement with Shimamoto et al. (1998) who showed that CYP2B activity was depressed by 54% 24 hours after an i.c.v. injection of 0.1 μg LPS. The discrepancy between doses of LPS (25 μg vs. 0.1 μg) most likely results from the different serotypes of LPS employed in these experiments. None of the acute phase cytokines, with the notable exception of IL-1β, could alter CYP2B1 activity in the liver. In contrast to the others, IL-1β significantly increased CYP2B1 activity by 42% over control levels. Whether this increase in hepatic CYP2B1 activity is a direct result of the actions of IL-1β or the result of an intermediate product induced by IL-1β remains to be determined.

Administration of LPS by i.p. injection significantly decreased CYP2B1 activity without a concomitant significant effect on protein levels. In a similar manner, animals treated with an i.c.v. injection of LPS also had significantly lower hepatic CYP2B1 activity. CYP2B1 protein levels appeared to be decreased in response to this treatment although statistical analysis could not be assessed due to a lack of sufficient sample size. It appears as though LPS, given i.c.v. or i.p. can down-regulate CYP2B1 activity in liver

through a pre-translational mechanism, although this must be confirmed with additional Western blots.

When cytokines were given by i.p. injection a significant decrease in CYP2B1 activity was seen for TNF-α only. In the case of IL-1β, no effect on CYP2B1 activity was found indicating that the effects seen with an i.c.v. injection may not be a direct result of IL-1β but depend on the generation of an intermediate product and/or activation of an additional system.

Examination of CYP2E1 activity in the livers of animals treated with an i.c.v. injection of drugs demonstrated that only LPS was capable of modulating CYP2E1 activity. 24 hours after injection of LPS (25 μg), hepatic CYP2E1 activity was decreased by 55%. Interestingly, the corresponding protein levels were found to be slightly, but not significantly, decreased (12%) and nowhere near the extent to which the activity was affected indicating that this response may result from post-translational modification or functional inhibition. In comparison, when drugs were injected i.p., both LPS and TNF-α were found to significantly decrease CYP2E1 activity. LPS and cytokines have previously been shown to decrease CYP2E1 when administered by i.p. injection and thus were used as positive controls for confirming the sensitivity of hepatic CYP2E1 activity to peripherally derived cytokines (Abdel-Razzak et al., 1993; Sewer et al., 1996). Protein levels for CYP2E1 in animals given 0.4 μg of TNF-α appeared to be decreased by 25% suggesting a possible effect on the synthesis of this isoform when the cytokine is administered peripherally. In contrast, CYP2E1 protein levels in animals that received an i.p. injection of LPS were not affected indicating a post-translational mode of regulation.

The results presented here demonstrate that centrally administered LPS decreases total hepatic cytochrome P450 content, CYP2B1 and 2E1 activity. We have also shown that centrally derived cytokines do not mediate this decrease in content or activity as i.c.v. injections of a variety of acute phase cytokines could not mimic the effects of LPS. In response to centrally administered LPS, significant amounts of cytokines are released in the periphery. These peripherally derived cytokines, and specifically TNF-α, may participate in the effects of an i.c.v. injection of LPS on cytochrome P450 enzymes in liver as this cytokine was shown to decrease total hepatic cytochrome P450 content, CYP2B1 and 2E1 activity when injected peripherally.

CHAPTER 7

NITRIC OXIDE AS A POTENTIAL MEDIATOR OF THE LIPOPOLYSACCHARIDE-INDUCED DEPRESSION IN CYP1A ACTIVITY

7.0 ABSTRACT

Down-regulation of cytochrome P450 isoforms by infection and inflammation is a well-documented event in liver and more recently in the brain. While the deleterious effects of altered levels of hepatic cytochrome P450 on overall drug metabolism has been established, much less is known about the consequences of altered cytochrome P450 levels in the central nervous system. It is thought that the expression of specific isoforms in the brain parenchyma may serve protective roles and disruption of this activity or expression may impact on this role.

Lipopolysaccharide is a commonly used immunostimulant that mimics the acute phase response. Recent work has demonstrated that this effect is partially mediated by the release of acute phase cytokines. Amongst the other immune mediators released during inflammation is NO. Since substantial quantities of this free radical are generated in response to LPS, it is possible that NO could mediate the down-regulation of CYP1A activity in cultured astrocytes.

This section examines NO as a potential mediator of the LPS induced down-regulation of CYP1A in cultured astrocytes. Results presented here found NO to mediate some of the effects of LPS on induced levels of CYP1A activity in astrocytes.

7.1 INTRODUCTION

Cytochrome P450 is a superfamily of heme containing enzymes that are primarily responsible for the metabolism and excretion of drugs from the body. Although the highest content of cytochrome P450 is found in the liver, isoforms do exist in various different tissues such as the skin, lungs, gastrointestinal tract, and, in recent years they have been found in the central nervous system (Norris et al., 1996). The function of these particular isoforms has not been elucidated as yet but they are thought to participate in blood vessel integrity and/or the protection of neurons from potentially toxic compounds (Kapitulink et al., 1987; Anandatheerthavarada et al., 1990). Recently, it has been described that cytochrome P450 isoforms within the brain are susceptible to regulation by infection or inflammation in the CNS (Tindberg et al., 1996; Nicholson and Renton, 1999; Renton et al., 1999; Nicholson and Renton, 2001c; Nicholson and Renton, 2001a). In response to trauma or infection, the CNS is capable of mounting a significant immune reaction (Montero-Menei et al., 1996). This response is characterized by activation of microglia, the resident macrophages of the brain, and the release of immune mediators such as cytokines, NO, and arachidonic acid metabolites (Boje and Arora, 1992; Sehic et al., 1996). Many of the important aspects of the immune response can be mimicked in experimental animals by injection of immune activators such as the bacterial endotoxin, LPS. When administered systemically, LPS can induce the release of acute phase cytokines, NO, and a variety of other mediators (Ziegler-Heirbrock, 1995). The release of some of these mediators, especially in the case of NO, results from induction of enzymatic activity. LPS can increase the expression and activity of inducible nitric oxide

synthase (iNOS) resulting in the synthesis and release of substantial quantities of NO (Cunha et al., 1994).

Current evidence has shown that LPS has similar effects when injected into the brain (Stern et al., 2000). Many of the mediators produced by LPS such as cytokines, and NO exert effects in the brain that are similar to those in the periphery. Centrally administered LPS increases the expression of inducible nitric oxide synthase in immunocompetent cells such as microglia resulting in the synthesis and release of NO within the brain which is bactericidal and functions as an important part of the inflammatory response (Garthwaite and Boulton, 1995).

The effects of infection or inflammation on hepatic cytochrome P450 have been well documented and shown to result from the generation of immune mediators following exposure to an inflammatory stimulus such as LPS (Ghezzi et al., 1986c; Morgan, 1993; Morgan et al., 1994; Muntané-Relat et al., 1995; Paton and Renton, 1998). Recently, similar effects have been shown to occur with cytochrome P450 enzymes within the brain parenchyma following an injection of LPS into the lateral ventricle (Shimamoto et al., 1998; Renton et al., 1999; Shimamoto et al., 1999; Nicholson and Renton, 2001c). It has been shown that cytokines play a role in the LPS-induced depression of CYP1A activity in the brain both *in vivo* and *in vitro*, however, they do not appear to be the sole mediators of this response. Other factors such as NO could potentially participate in this response.

This section examines the role of iNOS and the resulting production of NO on the loss of CYP1A evoked by LPS in cultured astrocytes. The evidence presented here demonstrates a partial role of NO in the depression of CYP1A activity in astrocytes.

7.2 MATERIALS AND METHODS

The culturing procedures and drug treatments were carried out as described in chapter 2.

7.3 RESULTS

The decrease in CYP1A activity mediated by LPS is accompanied by a concomitant release of NO. When astrocytes were incubated for 24 hours with LPS a concentration dependent decrease in EROD activity occurred and this was accompanied by the generation of substantial quantities of NO in these cultures (Fig. 7-1). Levels of EROD activity are depressed by 50, 70, 84, and 90% at doses of 5, 15, 25, and 37.5 μg/ml LPS respectively. Levels of NO as measured by nitrite production, increased from 4.3±3.8 nmol. NO_2^-/mg protein in control cultures to 45.0±5.8, 46.9±4.7, 38.6±5.4, and 34.7±4.5 nmol. NO_2^-/mg protein in those treated with 5, 15, 25, and 37.5 μg/ml of LPS respectively. For all subsequent experiments a concentration of 25 μg/ml LPS was utilized.

LPS induces the expression and activity of iNOS in cultured astrocytes. When astrocytes were incubated with 25 μg/ml LPS for 24 hours the expression of iNOS was considerably enhanced as determined by immunohistochemistry. Substantial iNOS staining was observed in cells from cultures treated with LPS compared to those that received saline only as illustrated in figure 7-2A&B. No staining was observed in the negative (no primary antibody) controls.

FIGURE 7-1

LPS Induces a Concentration-dependent Decrease in EROD Activity in Isolated Astrocytes. Cells were incubated with LPS for a period of 24 hours prior to assessing EROD activity and nitric oxide levels (n=4 plates per treatment). The solid bars represent CYP1A activity as measured by EROD activity (pmol. resorufin/mg prot./min.) whereas the solid line represents the amount of NO present in the culture medium of each treatment as measured by levels of nitrite (amount of nitrite $x10^{-1}$ nmoles/mg protein). Statistical significance was assessed using a one-way ANOVA with Student Newman-Keuls, $p<0.05$.

*significantly different from control
#significantly different from control

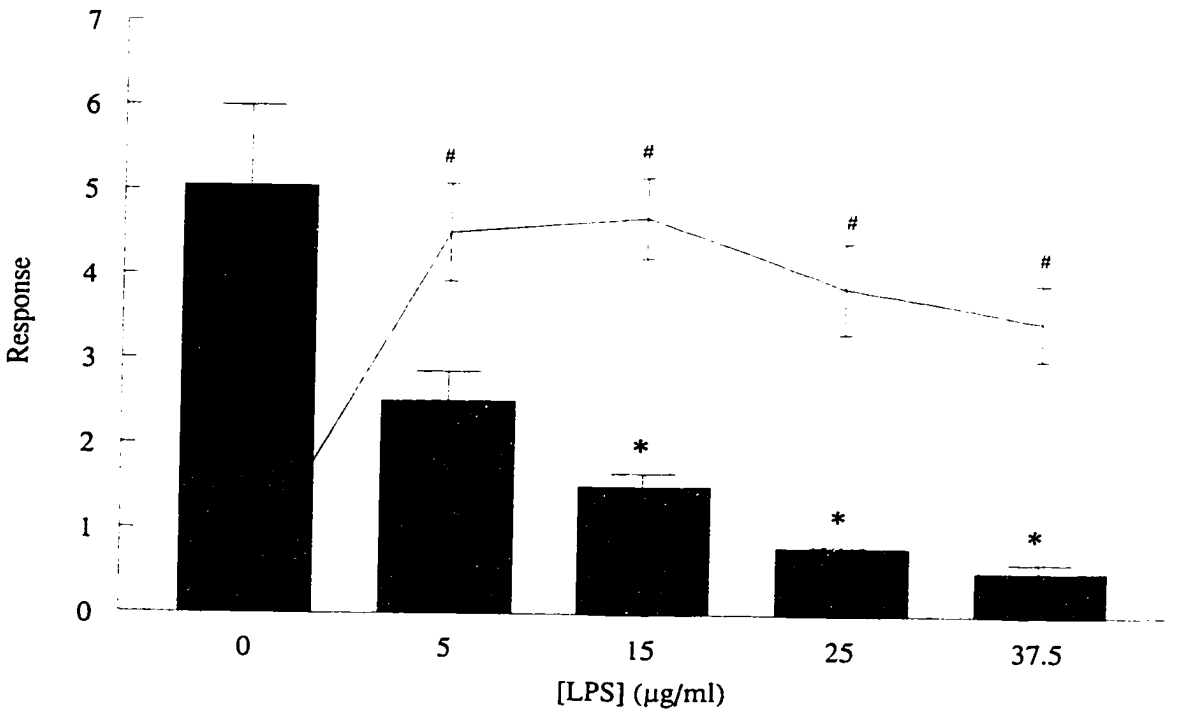
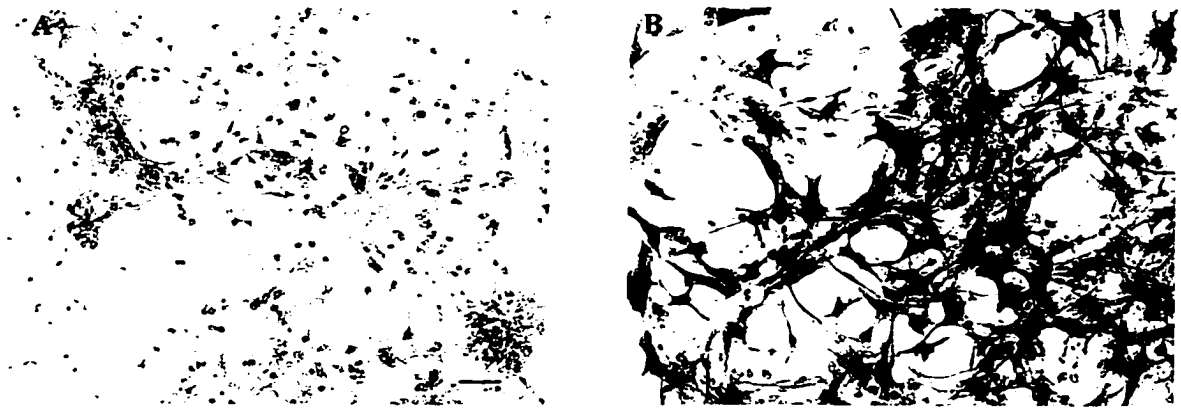


FIGURE 7-1

FIGURE 7-2

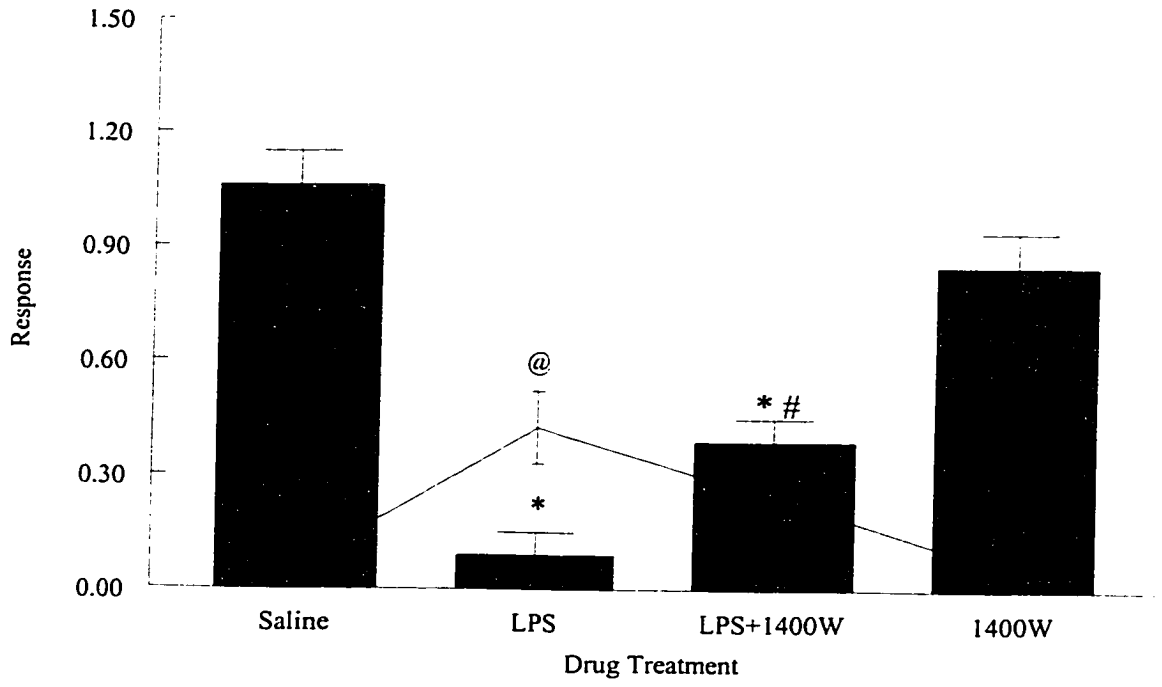


Isolated Astrocytes Respond to LPS through the Induction of iNOS Protein. Astrocyte cultures were incubated with saline or 25 μg/ml LPS for 24 hours then fixed in methanol and processed for immunohistochemical analysis of iNOS expression. Cultures that received saline showed little staining **(A)** whereas incubation with 25 μg/ml LPS led to robust staining for iNOS protein **(B)**. Scale bar=100 μm.

The selective iNOS blocker, 1400W partially attenuates the LPS induced depression of CYP1A activity and NO release. To determine whether the release of NO is a requirement for the LPS mediated decrease in CYP1A activity, cells were cultured with LPS and the selective iNOS blocker, 1400W. Cultures were pre-incubated with the inhibitor for 24 hours prior to addition of either saline or LPS. EROD activity was decreased by 92% in cultures incubated with LPS alone (Fig. 7-3). The addition of 1400W, caused a significant attenuation in the LPS induced depression in EROD activity whereas cultures incubated with 1400W alone had only a slight decrease in EROD activity. In cultures exposed to LPS alone a significant increase in NO was observed relative to those incubated with saline only. The amount of NO generated by LPS in cells treated with 1400W was reduced by 43% (Fig. 7-3).

Administration of sodium nitroprusside could not mimic the effects of LPS on astrocytes. In an effort to determine whether NO was one of the mediators of the LPS induced decrease in CYP1A activity, the nitric oxide donor, sodium nitroprusside (SNP), was added to isolated astrocytes to determine if this would emulate the effects of LPS. SNP did not have a significant effect on CYP1A activity in these cells at concentrations of 1, 10 or 100 μM SNP (Fig. 7-4). Incubation of cells with this drug results in increased nitrite levels in treated cultures, indicating that the drug did effectively release NO into the culture medium. Higher concentrations of SNP (up to 250 μM) were incubated with astrocytes, however, cellular toxicity limited these studies (data not shown).

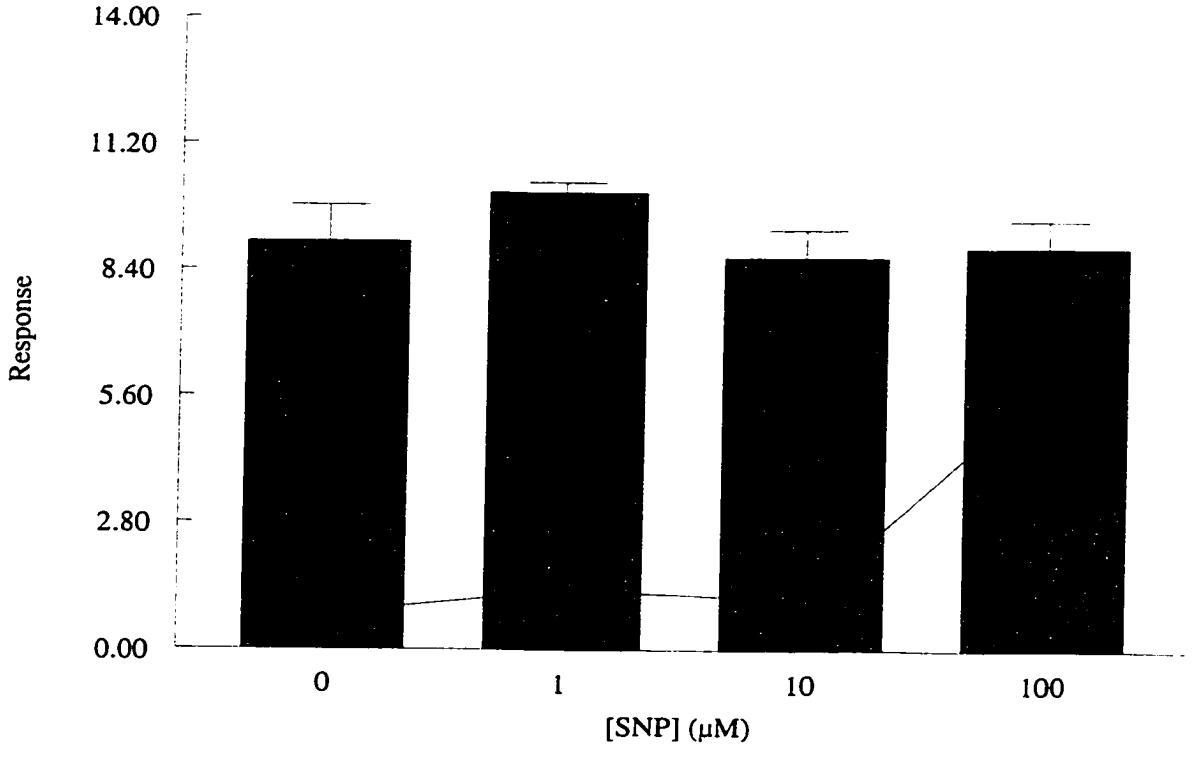
FIGURE 7-3



Blocking iNOS Activity Partially Attenuates the Effects of LPS on CYP1A Activity in Isolated Astrocytes. Astrocyte cultures that received 1400W were pretreated with the inhibitor for 24 hours before the addition of LPS or saline and a fresh dose of 1400W. The solid bars represent EROD activity (pmol. resorufin/mg prot./min) and the solid line, NO release (amount of nitrite $x10^{-2}$ nmoles/mg protein) (n=4 plates per treatment). Statistical significance was assessed using a one-way ANOVA with Student Newman-Keuls, $p<0.05$.

*significantly different from saline control.
#significantly different from LPS treated.
@significantly different from saline control.

FIGURE 7-4



SNP does not Mimic the Effects of LPS on CYP1A Activity in Isolated Astrocytes. Astrocytes were incubated with increasing concentrations of SNP for 24 hours (n=4 plates per treatment). The solid bars represent EROD activity (pmol. resorufin/mg protein/min.) and the solid line represents the NO levels (amount of nitrite $x10^{-1}$ nmoles/mg protein). Statistical significance was assessed using the one-way ANOVA with Student Newman-Keuls, $p<0.05$.

*significantly different from control.

7.4 DISCUSSION

The effects of infection or inflammation on the expression and activity of cytochrome P450 isoforms in the brain has been recently described (Renton et al., 1999; Nicholson and Renton, 2001c). Effects on cytochrome P450 expression are in part dependent on the release of proinflammatory cytokines from immunocompetent cells such as activated microglia and reactive astrocytes (Nicholson and Renton, 2001c). These results are not entirely surprising, as cytokines are known to decrease cytochrome P450 in the periphery (Ghezzi et al., 1986b; Ghezzi et al., 1986c; Morgan, 1993). However, in the brain, cytokines are not the sole mediators of this effect and thus the role of another immune mediator has been explored in this section.

When isolated astrocytes were incubated with LPS, a concentration-related depression in CYP1A activity was seen. Coincident to this decrease in CYP1A activity, there was an increase in NO level in culture medium of these treated cells. The intense staining for iNOS protein in the isolated cells reflected this increase in NO release. These observations are in agreement with others who have shown that LPS induces the activation of iNOS, and demonstrates why this molecule has been under intense scrutiny as a potential mediator of the LPS induced down-regulation of cytochrome P450 enzymes (Garthwaite and Boulton, 1995).

Much controversy has surrounded the role of NO as a mediator of LPS' effects on cytochrome P450 activity and expression. In some studies, a potential role for NO in mediating these effects has been demonstrated whereas in others, an independence of this response from NO release was shown (Khatsenko et al., 1993; Stadler et al., 1994; Carlson and Billings, 1996; Khatsenko and Kikkawa, 1997; Sewer and Morgan, 1997;

Takemura et al., 1999). Many of these effects depend highly on the isoform examined and the model utilized in the study. These limitations in interpreting results contribute greatly to controversy of NO and its role in this effect.

The *in vitro* studies reported here indicate a potential role for NO to partially explain the decrease in CYP1A activity in astrocytes. Evidence for a partial role of NO arises from results with the selective iNOS blocker, 1400W and its ability to only partially prevent the decrease in CYP1A activity mediated by LPS. This is not wholly surprising, as blockade of iNOS activity would have no bearing on cytokines, which modulate CYP1A activity in a NO-independent manner (Cunha et al., 1994; Nicholson and Renton, 2001a). SNP, the NO donor, did not affect CYP1A activity at any of the concentrations examined. This lack of response may indicate that NO is incapable of mediating decreases in CYP1A activity alone, but may require the presence of other immune substances normally released by LPS.

Taken together, evidence provided here suggests a potential role for NO in mediating the effects of LPS on centrally derived CYP1A activity. Clearly though, NO does not mediate these effects alone but is part of a complex interplay of immune mediators released during an inflammatory reaction.

CHAPTER 8

SERUM FROM ANIMALS WITH A CENTRAL INFLAMMATORY RESPONSE DEPRESSES CYP1A ACTIVITY IN CULTURED ASTROCYTES

8.0 ABSTRACT

The interaction and modulation of the cytochrome P450 system by infection and inflammation is a well-documented phenomenon. Recently, similar effects have been reported in animal models of central inflammation induced by administration of LPS into the lateral ventricle of the rat. This inflammatory response results in the depression of CYP1A activity in brain and CYP1A2 activity in liver. The mechanism by which these effects occur is suggested to be the release of cytokines in the brain and in the periphery. In the experiments described here, an *in vitro* model was utilized to demonstrate the effects of serum obtained from animals with an experimentally induced inflammatory response within the CNS.

The results presented here demonstrate that serum from animals given an i.c.v. injection of LPS can depress CYP1A activity in cultured astrocytes, implicating a serum factor as the mediator of these effects. Serum from animals that received a co-injection of dexamethasone did not depress CYP1A activity in astrocytes and had significantly reduced levels of cytokines in the serum obtained from these animals. This suggests a role for endogenously produced cytokines as potential mediators of this response.

8.1 INTRODUCTION

Cytochrome P450 expression is found in a variety of areas throughout the body including the liver, adrenal glands, lungs, GI tract, and in discrete areas of the brain (Norris et al., 1996). Recent evidence has shown that a central inflammatory response results in the modulation of cytochrome P450 activity in brain with a concomitant loss of cytochrome P450 activity in the liver (Shimamoto et al., 1998; Renton et al., 1999; Shimamoto et al., 1999; Renton and Nicholson, 2000; Nicholson and Renton, 2001c). Evidence has been presented to suggest that the effects on brain CYP1A activity result from the local production of cytokines within the brain parenchyma (Nicholson and Renton, 2001c). In contrast, the effects in liver do not seem to be mediated by the production of cytokines within the brain parenchyma, activation of the HPA axis, nor the stimulation of the sympathetic nervous system (Shimamoto et al., 1998; Shimamoto et al., 1999; Renton and Nicholson, 2000; Nicholson and Renton, 2001c). However, a role for peripherally derived cytokines has been suggested (Nicholson and Renton, 2001c). Cultured astrocytes have been used as an *in vitro* model to examine the direct effects of immune modulators such as LPS, cytokines, and NO on induced levels of CYP1A activity (Nicholson and Renton, 1999; Nicholson and Renton, 2001a; Nicholson and Renton, 2001b). *In vivo* experiments have suggested a role for cytokines in the down-regulation of CYP1A activity in brain and hepatic CYP1A2 activity during an LPS-induced central inflammatory response (Nicholson and Renton, 2001c). *In vitro* experiments have demonstrated that cytokines and NO may contribute to modulation of CYP1A activity in astrocytes (Nicholson and Renton, 2001a; Nicholson and Renton, 2001b). In this section we report that serum obtained from animals treated with an i.c.v.

injection of LPS or PolyIC can decrease induced levels of CYP1A activity in cultured astrocytes. These results suggest that it is a serum factor, possibly cytokines, produced during central inflammation that are responsible for mediating the decrease in CYP1A activity.

8.2 MATERIALS AND METHODS

All procedures were carried out as described in chapter 2.

8.3 RESULTS

Serum from LPS treated animals decreases CYP1A activity in astrocytes. Animals were injected i.c.v. with saline or 25 μg of LPS, sacrificed 5 hours later at the peak time for cytokine release, and blood collected. Serum obtained from these blood samples (either 50 or 100 μl) was incubated with cultured astrocytes for a period of 24 hours. EROD activity was not altered with a 50 μl dose of serum from LPS treated animals but was significantly decreased by 30% when 100 μl of serum was added to astrocyte cultures concomitantly with the CYP1A inducer DBA (50 nM) (Fig. 8-1). Cytokine levels were determined to be 161.7, 308.5, and 1732.9 pg/ml for TNF-α, IL-1β, and IFN-γ respectively in serum from LPS treated animals whereas animals that received an i.c.v. injection of saline had no detectable levels of cytokines (Table 8-1).

Serum from animals treated with DEX in combination with LPS does not decrease CYP1A activity in cultured astrocytes. Animals were treated with an i.p. injection of saline or DEX (0.4 mg) in combination with an i.c.v. injection of saline or LPS (25 μg).

FIGURE 8-1

Serum from LPS Treated Animals Depresses CYP1A Activity in Cultured Astrocytes. Serum was taken from animals 5 hours after receiving a 25 μg i.c.v. injection of LPS or saline. The serum obtained from these blood samples was incubated with astrocyte cultures for 24 hours then CYP1A activity assessed by measuring EROD activity (n=4 plates per treatment). Statistical significance was determined using the one-way ANOVA with Student Newman-Keuls.

*significantly different from saline control.

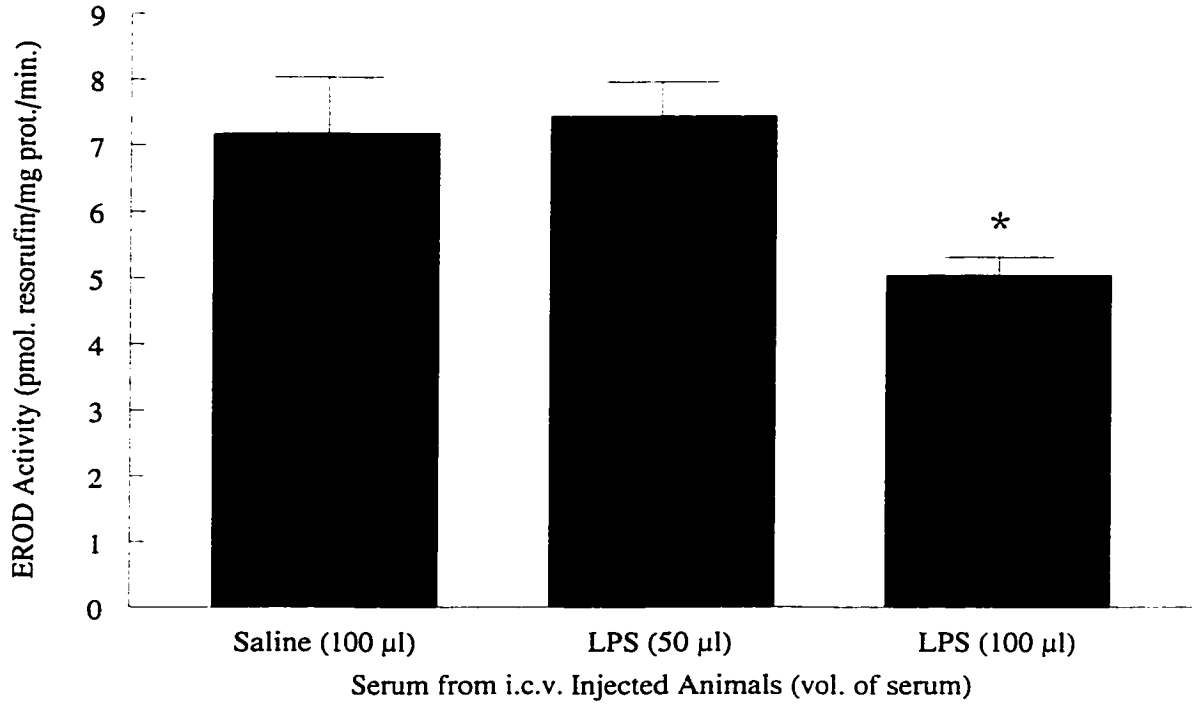

EROD Activity (pmol. resorufin/mg prot./min.)
9
8
7
6
5
4
3
2
1
0
*
Saline (100 µl)
LPS (50 µl)
LPS (100 µl)
Serum from i.c.v. Injected Animals (vol. of serum)

FIGURE 8-1

TABLE 8-1

Levels of cytokines in serum samples from treated animals and the resulting concentrations in culture medium.

Treatment (i.c.v.)	TNF-α (pg/ml)		IL-1β (pg/ml)		IFN-γ (pg/ml)	
	Serum	[Actual]	Serum	[Actual]	Serum	[Actual]
Saline	0	0	0	0	0	0
LPS	161.7	1.6†	308.5	3.1	1732.9	17.3
	161.7	3.2‡	308.5	6.2	1732.9	34.6

† 50 µl of serum from LPS treated animals was added to each plate of astrocytes (5 ml volume).

‡ 100 µl of serum from LPS treated animals was added to each plate of astrocytes (5 ml volume).

Blood samples were obtained from animals 5 hours after drug administration. 200 μl of serum was added to culture plates and incubated for 24 hours prior to measuring EROD activities. Activity levels in cultures that received serum from animals treated with saline-LPS (i.p.-i.c.v.) were depressed by 28% compared to controls (saline-saline) (Fig. 8-2). In contrast, animals that received DEX-LPS were not different from controls but were significantly different from cultures treated with saline-LPS serum. Serum from saline-saline or DEX-saline treated animals did not contain any cytokines whereas serum from saline-LPS animals contained substantial levels of TNF-α, IL-1β, and IFN-γ (161.7, 308.5, and 1732.9 pg/ml respectively) (Table 8-2). In comparison, only IL-1β could be detected in any significant concentration in animals treated with DEX-LPS, and its concentration was reduced by 41% compared to LPS alone.

PolyIC serum decreased CYP1A activity in cultured astrocytes. To examine whether these effects occur during different types of immune stimulation or, are specific to LPS, another model was utilized. Animals were injected i.c.v. with the interferon α&β inducer, PolyIC (25 μg) and sacrificed 2 or 4 hours later. Blood samples were obtained, serum extracted from these samples, and 200 μl added to astrocyte cultures and incubated for 24 hours. EROD activity was decreased by 36 and 34% by serum from PolyIC treated animals (2 and 4 hours respectively) (Fig. 8-3). Cytokine levels were determined in serum samples and found to contain only IL-1β at a concentration of 72.2 and 140.8 pg/ml for samples at 2 and 4 hours respectively (Table 8-3).

FIGURE 8-2

Serum from Animals Treated with LPS in Combination with DEX does not Alter CYP1A Activity in Cultured Astrocytes. At 5 hrs post injection, blood was taken from animals treated with an i.c.v. injection of saline or LPS (25 μg/ml) in combination with either saline or DEX (0.4 mg) by i.p. injection. 200 μl of serum was added to astrocyte cultures (5 ml per plate) and incubated for 24 hours prior to measurement of EROD activity (n=4 plates per treatment). Statistical significance was determined by using the one-way ANOVA with Student Newman-Keuls, $p<0.05$.

*significantly different from saline control.
#significantly different LPS alone (Sal.-LPS).

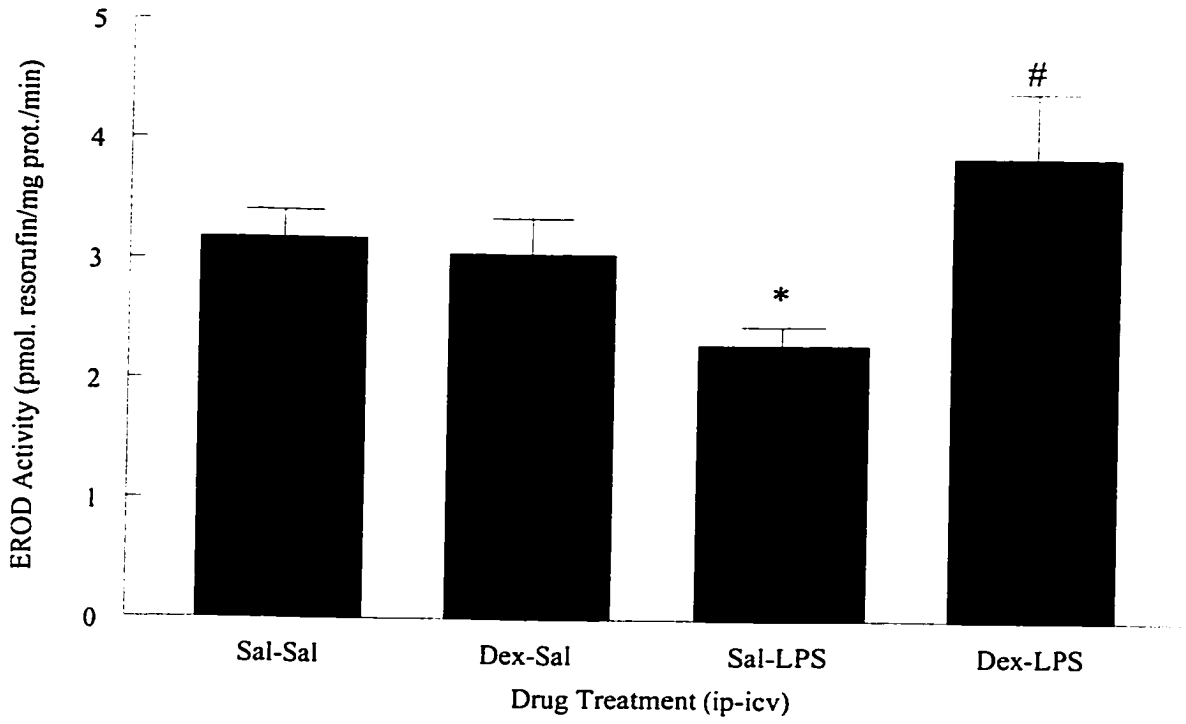

5
4
3
2
1
0
EROD Activity (pmol. resorufin/mg prot./min)
#
*
Sal-Sal
Dex-Sal
Sal-LPS
Dex-LPS
Drug Treatment (ip-icv)

FIGURE 8-2

TABLE 8-2

Cytokine levels in serum samples from treated animals and the resulting concentration in culture medium.

Treatment (i.p.-i.c.v)	TNF-α		IL-1β (pg/ml)		IFN-γ (pg/ml)	
	Serum	[Actual]†	Serum	[Actual]†	Serum	[Actual]†
Sal.-Sal.	0	0	0	0	0	0
DEX-Sal.	0	0	0	0	0	0
Sal.-LPS	161.7	6.5	308.5	12.3	1732.9	69.3
DEX.-LPS	0	0	184.0	7.4	0.01	0.0004

† 200 µl of serum from the different treatments was incubated with astrocyte cultures (5 ml volume).

FIGURE 8-3

Serum from Animals Treated with PolyIC Depresses CYP1A Activity in Cultured Astrocytes. Blood was taken from animals 2 & 4 hours after an i.c.v. injection of 25μg of polyIC. The serum extracted from the blood samples was incubated with cultured astrocytes for 24 hours then EROD activity determined as a measure of CYP1A activity (n=4 plates per treatment). Statistical significance was determined using the one-way ANOVA with Student Newman-Keuls, $p<0.05$.

*significantly different from saline control.

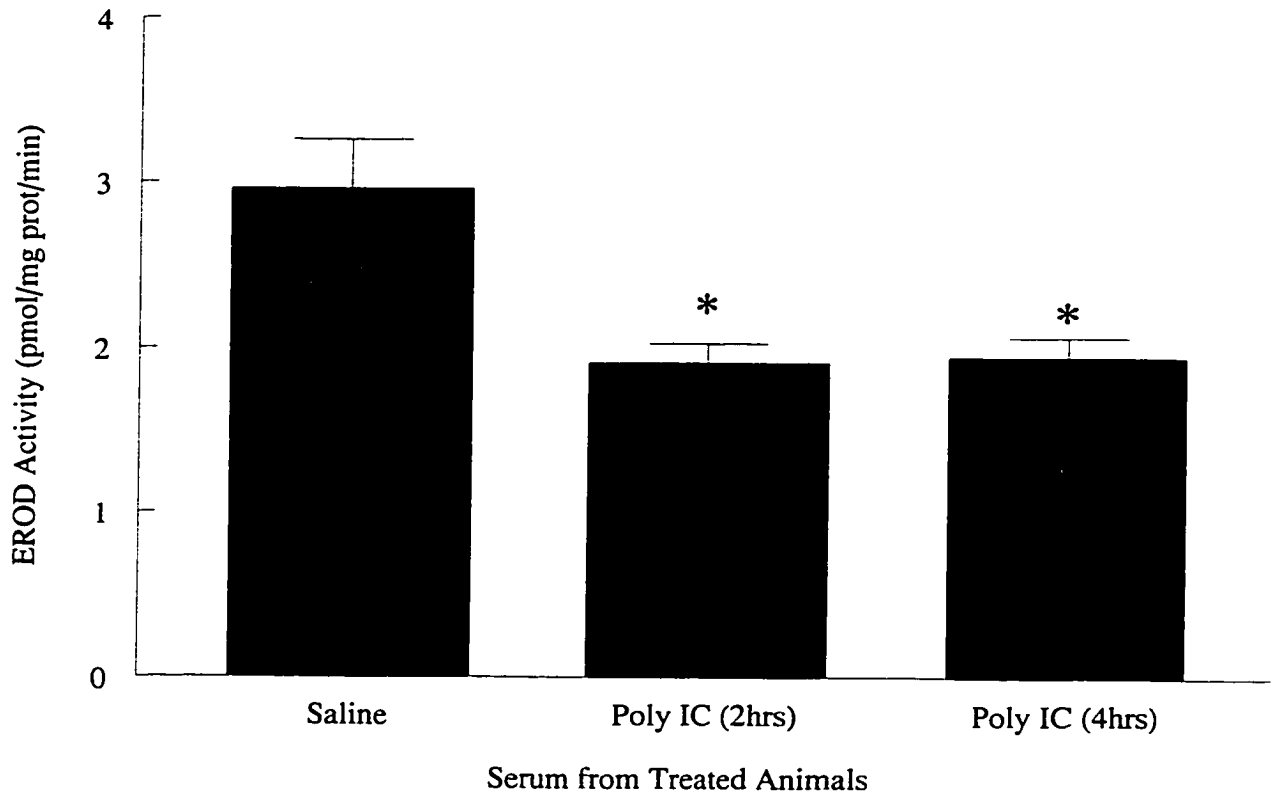

4
3
2
1
0
EROD Activity (pmol/mg prot/min)
*
*
Saline
Poly IC (2hrs)
Poly IC (4hrs)
Serum from Treated Animals

FIGURE 8-3

TABLE 8-3

Levels of cytokines in serum samples from PolyIC treated animals and the resulting concentrations in culture medium.

Treatment (i.c.v.)	TNF-α (pg/ml)		IL-1β (pg/ml)		IFN-γ (pg/ml)	
	Serum	[Actual]†	Serum	[Actual]†	Serum	[Actual]†
Saline	0	0	0	0	0	0
PolyIC (2hr)	0	0	72.2	2.9	0	0
PolyIC (4hr)	0	0	140.8	4.2	0	0

† 500 μl of serum from treated animals was incubated with astrocyte cultures (5 ml volume).

8.4 DISCUSSION

Modulation of cytochrome P450 activity by infection and inflammation is a well-characterized phenomenon with regards to the systemic system (Morgan, 1997; Iber et al., 1999). However, discovery of cytochrome P450 in the brain and the effects of a central infection or inflammatory response on cytochrome P450 in both the brain and the liver have sparked interest into the mechanism responsible for this effect and potential clinical implications. Current evidence has shown that cytokines are potential mediators of these effects both in the brain and in the periphery (Nicholson and Renton, 2001c). In the experiments reported here, serum from animals given an i.c.v. injection of LPS decreased CYP1A activity in cultured astrocytes. This implicates a serum borne factor as the mediator(s) of the decrease in CYP1A activity following a central inflammatory response. Levels of the acute phase cytokines TNF-α, IL-1β, and IFN-γ were determined in serum samples prior to incubation with astrocyte cultures. Interestingly, cytokine levels in serum from LPS treated animals were found to be in the low pg/ml range whereas previous work has shown that exogenously administered recombinant cytokines affect CYP1A activity in cultured astrocytes at substantially higher concentrations (~20 ng/ml). This discrepancy could indicate that cytokines are not the mediators of this response. The evidence presented here argues against this hypothesis as the concomitant injection of DEX (i.p.) with LPS (i.c.v.) led to substantially reduced cytokine levels in the serum of these animals and this serum did not depress CYP1A activity whereas serum from LPS treated animals did show a significant depression. Another feasible explanation would be that other serum mediators besides the cytokines TNF-α, IL-1β, and IFN-γ may be mediating these effects. Other cytokines such as IL-6, free radicals

such as nitric oxide (NO), and arachidonic acid metabolites such as PGE_2 could also be involved. Since these mediators are induced by acute phase cytokines such as TNF-α, this might explain why the serum from DEX-LPS treated animals did not depress CYP1A activity in astrocytes (Norris et al., 1994; Minghetti et al., 1999). Recently it has been shown that NO is not released in astrocyte cultures by TNF-α and IFN-γ while IL-1β will release low levels of NO but only in high doses (50 ng/ml) (Nicholson and Renton, 2001a). At the low cytokine concentrations utilized in this experiment it is unlikely that NO would be released in sufficient amounts. In addition, IL-6 has not been shown to have an effect on CYP1A activity when incubated with cultured astrocytes (Nicholson and Renton, 2001a) and neither has PGE_2 (unpublished observations). These results imply that although other mediators may be involved these do not seem to include NO, IL-6, or PGE_2. Lastly, the discrepancy between concentrations of cytokine utilized in these experiments versus earlier ones may result from adding each cytokine in isolation as many of their effects result from the synergistic interaction between different cytokines (Carlson and Billings, 1996; Paludan, 2000). In the case of serum from DEX-LPS treated animals, significant levels of IL-1β only could be detected in serum and this was found to be insufficient to affect CYP1A activity in cultured astrocytes.

I.c.v. injection of PolyIC, an interferon α&β inducer, was used as a model of a viral infection localized to the CNS. Serum extracted from these animals at 2 and 4 hours following injection depressed CYP1A activity in cultured astrocytes incubated with the serum for 24 hours. Serum from these PolyIC treated animals contained IL-1β in relatively low levels and is most likely, insufficient to alter CYP1A activity. Despite the low levels of cytokines, CYP1A activity was depressed by 36 and 34% in cultures that

were incubated with serum obtained at 2 and 4 hours after injection of PolyIC respectively. This decrease most likely results from the generation of IFNs (α and β) that are known to decrease CYP1A activity *in vivo* (Parkinson et al., 1982; Stanley et al., 1991).

The results presented here demonstrate that a serum factor(s) is produced in the periphery in response to an i.c.v. injection of LPS that is capable of decreasing CYP1A activity in cultured cells. It appears as though cytokines, and in particular a combination of cytokines, are important in mediating this response as blocking their release into serum abrogates the response in cultured astrocytes. In the case of PolyIC, the serum mediator(s) do not seem to be TNF-α, IL-1β, or IFN-γ as they are not present or found only in very low levels in serum from these animals. However, CYP1A activity is depressed in astrocytes cultures treated with this serum and this effect may be a result of IFN-α&β produced in response to PolyIC, although this must be confirmed by additional experiments. In addition, the effects of serum from i.c.v. treated animals needs to be examined on hepatocytes to confirm the susceptibility of CYP1A regulation in this physiologically relevant cell population.

CHAPTER 9

SUMMARY, SIGNIFICANCE AND FUTURE WORK

9.1 SUMMARY

The work presented in this thesis highlights important interactions between central infection and inflammation and the resulting effects on cytochrome P450 expression and activity. Cultured astrocytes were used as an *in vitro* model in which to study the direct effects of LPS and cytokines on induced CYP1A activity. These results demonstrate that acute phase cytokines such as TNF-α, IL-1β, and IFN-γ are capable of depressing CYP1A activity, however none of them could fully mimic the effects of LPS. Co-incubation of cultures with the cytokine inhibitor DEX blocked the LPS induced depression of CYP1A indicating that cytokines are indeed involved in this effect. Similarly, NO was shown to have a small role in mediating these effects as co-incubation of LPS with the selective iNOS blocker, 1400W partially reversed the effect of LPS on CYP1A activity. Taking these results into consideration, it is likely that the effects of LPS are due to the production of several immune mediators including but not limited to the release of acute phase cytokines, free radicals, and arachidonic acid metabolites.

Cultured astrocytes provide a simplified system in which to assess the role of immune mediators. However, these results cannot take into consideration the interplay between different systems in the intact animal, for these reasons parallel experiments were carried out in the whole animal. These results complemented those found using the cultured astrocytes and provided interesting insights into the differential effects of cytokines in this *in vivo* model. The experiments highlighted in this thesis demonstrate that when cytokines were injected i.c.v. (a model of local cytokine production) CYP1A activity in brain was depressed while total hepatic cytochrome P450 content and levels of CYP1A2, 2B1, and 2E1 activity in liver were not affected with the exception of an

increase in CYP2B1 activity in response to an i.c.v. injection of IL-1β. Cytokines administered by the i.p. route revealed that total cytochrome P450 content and the associated enzymatic activities (CYP1A2, 2B1, and 2E1) in the liver are susceptible to regulation by cytokines (in particular TNF-α). Analysis of cytokine levels in serum samples obtained from the periphery following an i.c.v. injection of LPS showed a particularly interesting result; animals injected with LPS had high levels of acute phase cytokines TNF-α, IL-1β, IL-6, and IFN-γ whereas animals injected (i.c.v.) with saline, TNF-α, or IL-1β had no significant levels of these cytokines. These results suggest that peripherally derived cytokines play a role in the down-regulation of cytochrome P450 in liver following an i.c.v. injection of LPS. It was interesting to note that three different isoforms of cytochrome P450 were differentially affected by peripherally administered cytokines. All isoforms were susceptible to TNF-α (i.p.) whereas only CYP1A2 activity was altered by IFN-γ (i.p.).

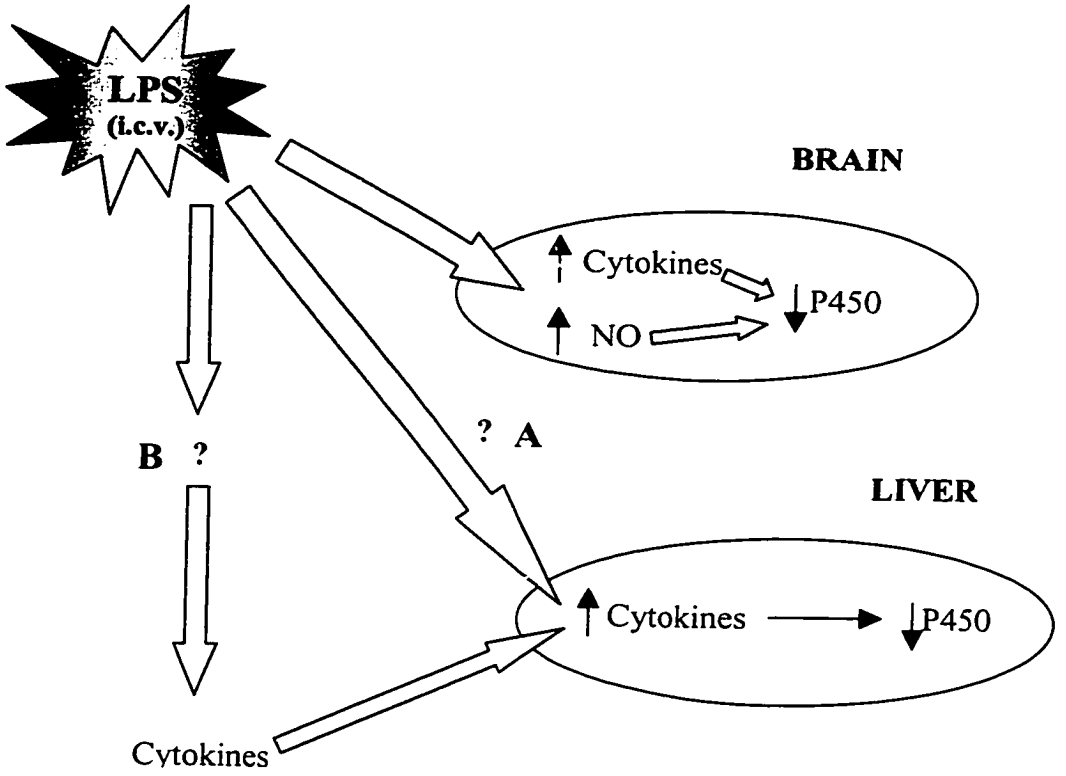
Astrocyte cultures were used to confirm that a serum factor(s) is responsible for the peripheral effects of LPS (i.c.v.) on CYP1A activity. Serum was extracted from rats 5 hours after an i.c.v. injection of LPS, the peak time point for cytokine release. Significant concentrations of TNF-α, IL-1β, and IFN-γ were detected in this serum sample and, when added to astrocyte cultures, decreased CYP1A activity implicating cytokines as participants in this response. In serum from animals treated with LPS (i.c.v.) and DEX (i.p.) levels of cytokines were significantly reduced and this serum did not modulate CYP1A activity thus supporting the hypothesis that cytokines are the serum mediators involved in the depression of cytochrome P450 activity following central administration of LPS.

This thesis provides evidence for a role of cytokines and possibly NO in mediating the local effects of LPS on CYP1A activity in the brain. These centrally produced cytokines do not appear to directly mediate the effects on liver cytochrome P450, however, peripherally released cytokines do appear to participate in the regulation of total hepatic cytochrome P450 content and the activity of the CYP1A2, 2B1, and 2E1 isoforms. A hypothetical cascade for the effects of centrally administered LPS on cytochrome P450 expression and activity is shown in figure 9-1.

9.2 SIGNIFICANCE

This work focuses on the interaction between the immune and drug metabolizing systems of the body. It is particularly interesting that the system that affords us protection from infectious agents should modulate the system that protects us from chemical insults. A consistency of this effect is seen across species and in different models of infection and/or inflammation suggesting this is a physiologically important interaction (Chang et al., 1978; Shedlofsky et al., 1987; Moochhala, 1991; Morgan, 1993; Shedlofsky et al., 1994; Renton and Nicholson, 2000). Several hypotheses have been suggested in this regard and are reviewed by Morgan (2001). The dedication of transcriptional machinery to the synthesis of immune mediated products such as acute phase proteins has been suggested as one of these possible hypotheses. However, this does not adequately explain why cytochrome P450 isoforms in extra-hepatic locations are affected or why the various isoforms are differentially regulated. Presumably, if this type of regulation occurred all isoforms would be affected to the same degree and this is clearly not the case (Stanley et al., 1991; Sewer et al., 1996). Another possible

FIGURE 9-1



Hypothetical Scheme for the Mechanism by which LPS Affects CYP1A Activity. I.c.v. injection of LPS induces the release of several immune mediators within the brain of which acute phase cytokines and NO have been shown to alter CYP1A activity in astrocytes. The effects of LPS on liver CYP1A2 activity seem to involve the induction of cytokine release within the periphery. This peripheral release of cytokines may be a direct result of LPS action within the brain **(A)**, or could possibly arise from the stimulation of an intermediate system **(B)**.

explanation for this interaction involves the generation of reactive oxygen species. The uncoupling of cytochrome P450 reactions (specifically with CYP2E1) results in the generation of significant quantities of free radicals. These compounds can interact with reactive nitrogen species (NO) generated by the immune system resulting in the formation of the highly reactive compound peroxynitrite. This compound can seriously disrupt normal cell function, an effect that is particularly important in brain due to its limited regenerative capacity (Wood, 1998).

Although the effects of a systemic infection or inflammatory response on cytochrome P450 are well documented, the observation that neuroinflammation and/or central infection can also modulate cytochrome P450 activity within the brain and the liver is a more recent observation (Shimamoto et al., 1998; Renton and Nicholson, 2000; Nicholson and Renton, 2001). These interactions may be particularly important in patient populations who have an acute infection such as meningitis, acute brain trauma, or a chronic inflammatory condition such as those seen in the neurodegenerative diseases, MS, Parkinson's, and Alzheimer's disease. These short-term or long-term conditions can result in the disruption of the normal physiological role of cytochrome P450 in the brain and the liver. The functional consequences of altering brain cytochrome P450 activity are not fully characterized but may include interference with the synthesis of endogenous compounds such as neurosteroids, and/or have effects on cytochrome P450 isoforms within the BBB that are thought to prevent the entrance of potentially toxic compounds into the brain (Mesnil et al., 1984; Majewska et al., 1986; Stromstedt et al., 1993; Morse et al., 1998). The down-regulation of cytochrome P450 isoforms in liver during central infection and/or inflammation can have serious toxicological implications particularly for

patients on drugs with a narrow therapeutic index as metabolic inhibition will increase plasma levels of these drugs. Equally problematic is the effect this would have on pro-drugs that must be metabolically activated. Disruption of cytochrome P450 activity in this case could prevent the therapeutic effects of the drug.

Clearly, the interaction between central infection and/or inflammation and the cytochrome P450 system must be characterized and the extent of this interaction determined in susceptible patient populations. This thesis was designed to investigate the mechanism behind this interaction and reveal potential points of manipulation to prevent this potential complication within patient populations that are at risk for such interactions.

9.3 FUTURE WORK

1. Elucidate the detailed molecular mechanism by which cytokines and/or NO modulate the expression and activity of cytochrome P450 enzymes in the liver.

The experiments described here demonstrate that centrally administered LPS induces the release of peripheral cytokines. The mechanism by which this occurs and the source of these cytokines remains unclear and need to be addressed in future experiments such as those highlighted below.

2. To investigate the effects of cytokine blockers on centrally administered LPS.

Results from this thesis implicate locally produced cytokines as mediators of the effects on cytochrome P450 in the brain, and peripherally derived cytokines in mediating the effects on liver. However, these studies cannot definitively reveal whether the

cytokines are the causative agent of this effect. I.c.v. injection of ICE (interleukin-1β converting enzyme) inhibitors will assess the role of centrally produced IL-1β in this response while i.p. injection of ICE inhibitors can be used to assess the role of peripherally released IL-1β. In a similar vein, antibodies to other cytokines such as TNF-α and IFN-γ can be utilized to examine their roles in this effect.

3. To assess the effects of cytokines and other immune mediators given in combination both *in vivo* and in astrocyte cultures.

LPS administration either *in vivo* or *in vitro* stimulates the release of a large variety of immune mediators including cytokines, free radicals, and prostaglandins. Since these mediators are released in consort, it is important to assess their effects when given in combination. Administration of cytokine combinations in conjunction with NO or PGE_2 may be able to fully mimic the effects of LPS when given i.c.v. as the effects of this endotoxin no doubt involve a complex interplay of these mediators.

4. To identify relevant drug interactions within susceptible patient populations and investigate the nature of these interactions.

In collaboration with clinical investigators, it would be informative to identify patients with chronic neuroinflammatory conditions and assess drug interactions within this population. In addition, *in vivo* metabolism studies could be performed using probe substrates such as caffeine (CYP1A2) and debrisoquine (CYP2D6) on these patients and compared to age matched controls (note: investigation into CYP2D6 would require taking into consideration genetic polymorphisms). It would be particularly interesting to

assess *in vivo* drug metabolism in patients with MS as this disease is cyclic in nature and the comparison of drug metabolism during an acute attack vs. a period of remission would provide information as to the direct effects of neuroinflammation on drug metabolism.

APPENDIX A

Listing of the Antibodies used and their Applications

Application	Primary Antibody	Secondary Antibody
Western Blot	Goat anti-rat CYP1A1, 2E1, or 2B1	Anti-goat IgG conjugated to peroxidase (POD)
Immunofluorescence	Mouse anti-rat GFAP	Anti-mouse conjugated to TRITC
Immunofluorescence	Mouse anti-neurofilament 160	Anti-mouse conjugated to TRITC
Immunofluorescence	Goat anti-rat CYP1A1	Anti-goat conjugated to FITC
Immunohistochemistry	Horse anti-mouse CD11b/c (OX42)	Anti-mouse IgG conjugated to biotin (rat absorbed)
Immunohistochemistry	Rabbit anti-mouse hsp25	Anti-rabbit IgG conjugated to biotin
Immunohistochemistry	Rabbit anti-mouse iNOS	Anti-rabbit IgG conjugated to biotin

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