MEDIATORS OF THE DEPRESSION IN CYP1A ACTIVITY PRODUCED BY CYTOKINES

by

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ABSTRACT

Mediators of the Depression in CYP1A Activity Produced by Cytokines

Infections and other situations which result in the activation of host defense mechanisms, are known to result in a depression in cytochrome P450 mediated metabolism in the liver. Initially, it was observed that interferon (IFN) and IFN inducers depress various forms of cytochrome P-450 by decreasing the production of mRNA for the apocytochrome. It was later demonstrated that other cytokines, including interleukin 1 (IL-1) and tumor necrosis factor (TNF), are capable of producing the depression independent of IFN. The decrease in cytochrome P450 produced by *in vivo* administration of IFN can be prevented by the addition of a protein synthesis inhibitor, suggesting the involvement of an intermediate protein, produced in response to the cytokine. The identity of the intermediate protein has not been determined (Renton, 1994). This research further characterizes the protein in a primary hepatocyte model. A time course study suggests that the intermediate protein may be present as early as 6 hours after TNF stimulation. Protein synthesis inhibitors added to primary hepatocyte cultures prevented the depression in CYP1A activity in response to TNF. Nitric oxide has been proposed as a possible mediator of the depression of cytochrome P-450 in response to cytokines. The addition of nitric oxide generating compounds depressed CYP1A activity in this system, and a NOS2-selective inhibitor prevented the depression of CYP1A activity in response to cytokines. The possible involvement of NF-kB in cytochrome P-450 regulation was also investigated, with the use of selective inhibitors of NF-kB. In other cell types, TNF has been shown to be a potent activator of NF-kB. NF-kB is expressed in hepatocytes, where it can be activated in response to endotoxin stimulation (Essani, 1996). Experiments using the NF-kB inhibitors suggest that this transcription factor may have a role in cytochrome P-450 regulation.

ABBREVIATIONS AND SYMBOLS

Act D	Actinomycin D
BCIP	5-bromo-4-chloro-3-indolylphosphate
BNF	beta Naphthoflavone
BSA	bovine serum albumin
BTEE	N-benzoyl-tyrosine ethyl ester
CYP1A	Cytochrome P-4501A
DBA	dibenzanthracene
DMSO	dimethyl sulfoxide
DON	deoxynivalenol
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EROD	ethoxyresorufin-O-deethylase
FBS	fetal bovine serum
I-κB	inhibitor kappa B
ICAM	intracellular adhesion molecule
IL	interleukin
IFN	interferon
IP	intra peritoneal
KCM	Kupffer cell conditioned medium
KCM-DON	DON-treated-Kupffer cell conditioned medium
KCM-LPS	LPS-treated-Kupffer cell conditioned medium
L-NAME	NG-nitro-L-arginine methyl ester
LPS	lipopolysaccharide
NADH	nicotinamdie adenine dinucleotide (reduced form)
NADPH	nicotinamdie adenine dinucleotide phosphate (reduced from)
NBT	nitro blue tetrazolium
NF-κB	Nuclear factor kappa B
NIL	L-N6-(1-imino ethyl) lysine

NIO	L-imino ornithine
NMA	N-methyl-L-arginine
NNA	N-nitro arginine
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate buffered saline
poly IC	polyriboinosinic polyribocytidylic acid
Puro	puromycin
SDS	sodium dodecyl sulfate
SIN-1	3-morpholino-sydnonimine
SNP	sodium nitroprusside
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF	tumor necrosis factor
TPCK	N-tosyl-l-phenylalanine chloromethyl ketone
Tris HCL	tris[hydromethyl] aminomethane hydrochloride
Tween 20	polyoxyethyylenesorbitan monolaurate

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INTRODUCTION

1.1 THE LIVER

The primary function of the liver is to both regulate and process various substances found within the circulation. The liver receives blood from the splanchnic circulation, from which it takes up solutes that have been excreted by the pancreas, spleen, and intestine. It is also perfused by the systemic circulation. The functional unit of the liver is the acinus, which is a mass of cells which are unilaterally perfused with blood from these two circulatory systems. The hepatocytes, or parenchymal cells, form a single layer tube which are approximately 20-25 hepatocytes long. These cells are polarized, and possess a sinusoidal, or basolateral domain facing the sinusoidal space. Adjacent hepatocytes are connected through gap junctions, to establish communication between neighboring cells. The polarity of the hepatocyte is important for its overall function, and the disruption of this structure which occurs during primary cell culture could possibly contribute to the differences in function observed in isolated hepatocytes compared to the intact liver.

Within the space formed by the hepatocytes, are various other cell types of the liver (fig. 1). Fat storing cells (Ito cells), and fibroblasts lie next to the hepatocyte, in the perisinusoidal space, or space of Disse. Beyond this are the endothelial cells, which form the sinusoidal wall. These cells may be separated by spaces called fenestrations, through which particles and solutes can pass. Kupffer cells, which are monocytes, lie within the sinusoidal space fixed to the endothelial cells. They are the largest group of fixed

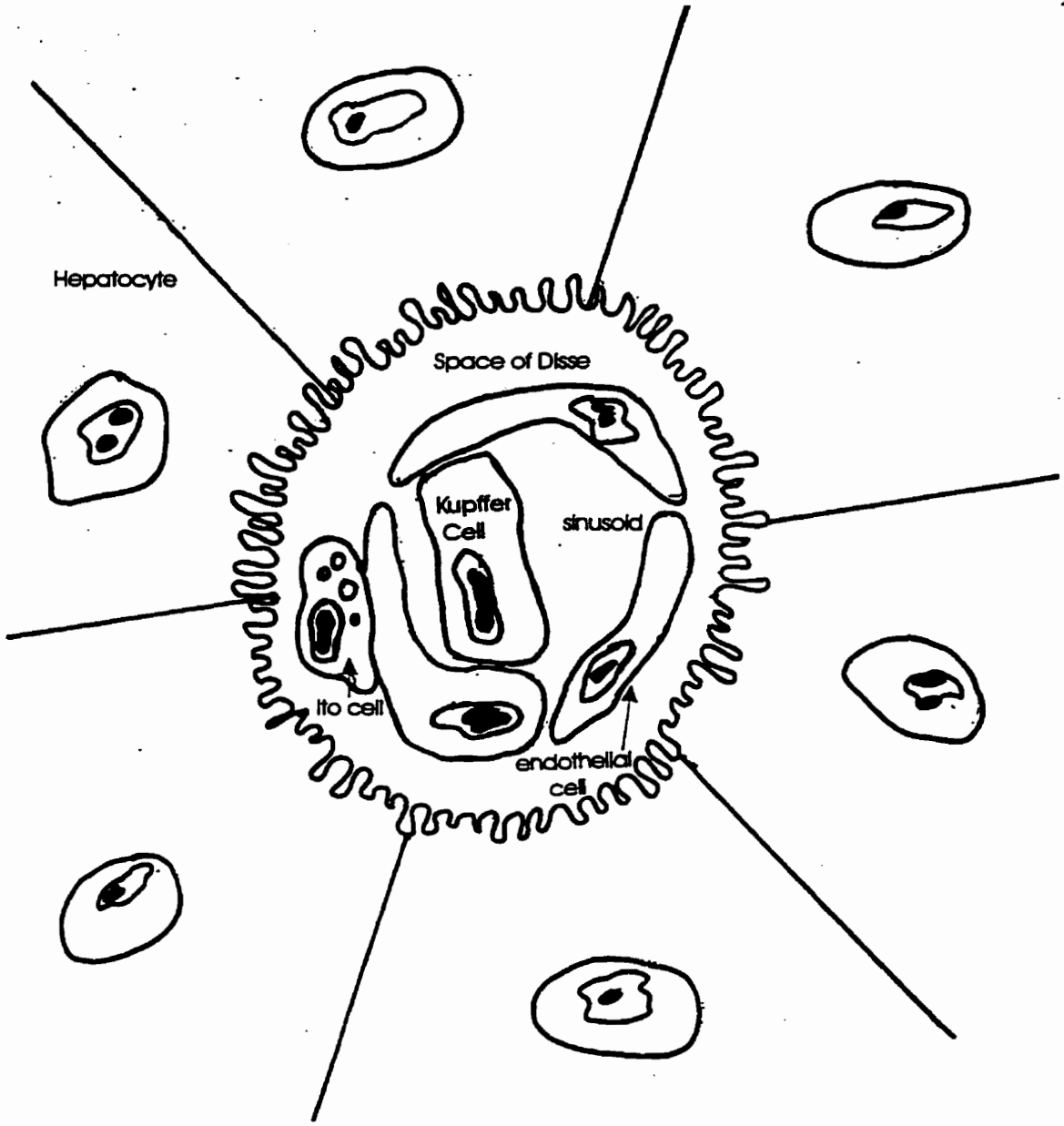


FIGURE 1

Structure of the hepatic acinus
This is a representation of the hepatic sinusoid, which portrays the relative positioning of the cells of the liver (adapted from Gumucio, 1992).

macrophages found within the body. Besides these cells, an important component of the liver is the extracellular collagen matrix on which it is structured.

Blood moves from the terminal portal venule and hepatic arteriole towards the hepatic venule. This results in the distribution of substances such that the hepatocytes closest to the portal venule receive a greater concentration than those located further towards the hepatic venule. The hepatocytes that form the acinus are not all uniform; they differ in their expression of genes and in their function from one end to the other. Most of the cytochrome P-450 enzymes (the topic of this thesis) are located in the distal two thirds of the hepatic acinus. The hepatocytes within the first three to four rows of hepatocytes of the proximal to the portal venule are able to undergo mitosis, and are responsible for cell renewal, but the hepatocytes beyond the fifth position lose this capacity. The last two rows of hepatocytes express glutamine synthetase and carbonic anhydrase II and III. The explanation for the differences in the hepatocytes within the acinus is not known, but it may be due to differences in the substances presented by the blood, or it may be due to different cellular connections. This structure is lost during primary hepatocyte culture, and may account for some differences in results between *in vivo* and *in vitro* work. (Gumucio et al, 1992)

1.2 CYTOCHROME P-450 SYSTEM

Cytochrome P-450 refers to a superfamily of enzymes, known as the mono-oxygenase system, which are responsible for the metabolism of a wide variety of endogenous and exogenous compounds. Cytochromes P-450 are heme containing enzymes, located on the rough endoplasmic reticulum, which contain one prosthetic heme (iron-porphyrin) moiety bound to an apoprotein (Halpert et al,1994). They are named for the spectral absorption maximum at 450 nm that occurs when the cytochrome is reduced and bound to carbon monoxide (Omura et al, 1962). This enzyme system catalyzes oxidation reactions, where lipophilic substrates are converted into more polar products which can be easily excreted. The overall effect of the catalytic cycle is to add one oxygen atom to the substrate (fig. 2). First, the substrate binds to the cytochrome P-450 enzyme, and then the iron of cytochrome P-450 is reduced by an electron supplied by the oxidation of the cofactor NADPH by cytochrome P-450 reductase. This reduced form of the cytochrome P-450-substrate complex binds a molecule of oxygen, and a second electron is then added by cytochrome P-450 reductase. In some cases this second electron may be supplied by the oxidation of the cofactor NADH by cytochrome b_5 reductase. This second electron activates the oxygen, so that it can interact with the substrate. Finally, one atom of oxygen is reduced to water, while the other oxygen atom is transferred to the substrate which is then released, as the oxidized product (deBethizy et al, 1994).

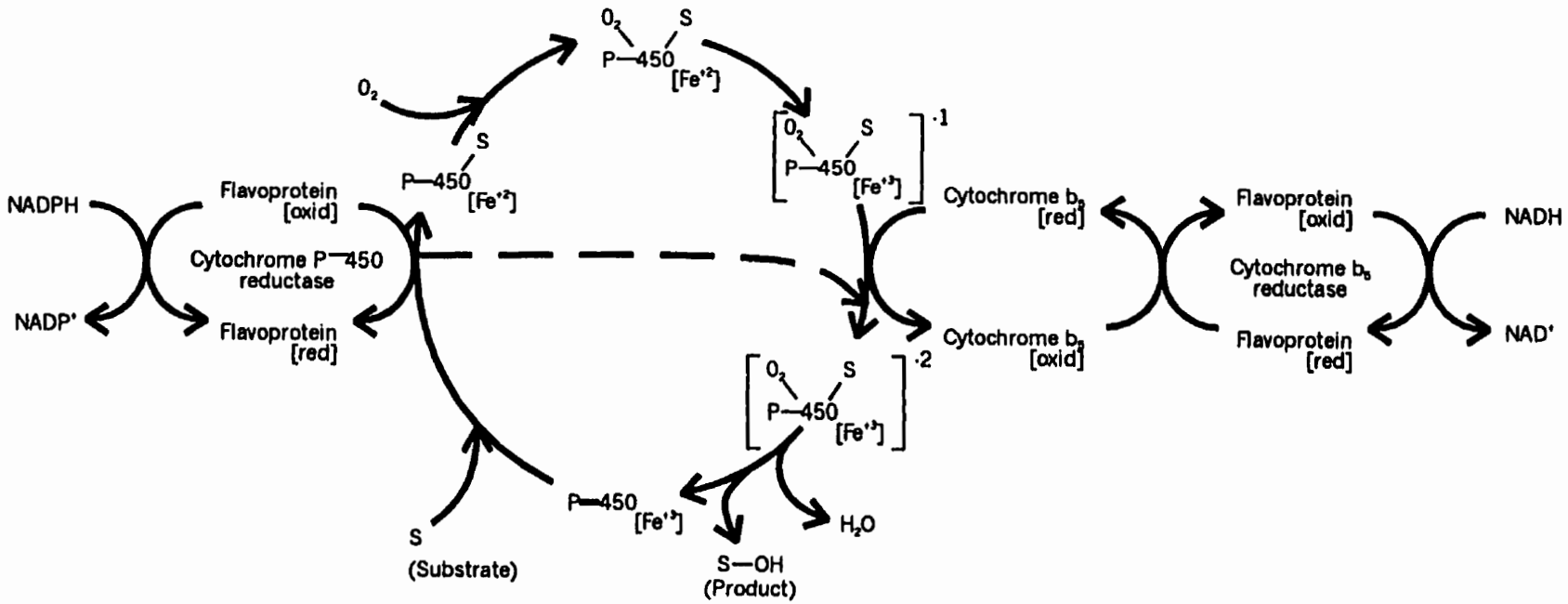


FIGURE 2

Catalytic cycle of the Cytochrome P-450 - dependent monooxygenase system. (adapted from deBethizy, 1994)

The system is able to handle a wide range of compounds, utilizing many relatively non-specific isozymes of cytochrome P-450. These are characterized and classified into at least 36 gene families based on structure, with members of the same family exhibiting at least 40% amino acid sequence homology. Many of the families are then further subdivided into sub families, where members share at least 55% sequence homology. Cytochromes P-450 are named by using the root "CYP", followed by a number to indicate the family, a letter to indicate the sub-family, and a number to indicate the specific form. (Gonzalez, 1992). For example in CYP2D6, 2 indicates the family, D indicates the sub-family, and 6 indicates the specific form.

Another factor which contributes to the diversity of this system is that although the liver contains the highest cytochrome P-450 activity, cytochromes P450 are also found throughout the body including central nervous system, intestine, lung, heart, and kidney. In the liver CYP1, CYP2, and CYP3 play a major role in the metabolism of xenobiotics, although other forms can metabolize specific drugs or chemicals (Halpert et al, 1994). The half life of drugs and toxins which are dependent upon cytochrome P-450 metabolism for elimination, are greatly effected by the rate at which this conversion to the polar, excretable form occurs. Therefore, the amount of the particular isozyme responsible for the reaction, therefore, largely determines the rate of metabolism. As the levels of an enzyme change in response to changes within the cellular environment, the rate at which the body can metabolize that enzyme's substrate(s) also changes, and this in turn results in a change in the half-life of many drugs and chemicals.

As cytochrome P-450 enzymes are responsible for such a broad range of metabolic

processes, and because their level and/or activity may change in response to their endogenous and exogenous stimuli, a number of both positive and negative interactions have been reported. Many drug interactions are due to changes in the rate of metabolism of one drug after exposure to a second drug. If a drug induces an increase in expression of a particular isozyme, other drugs metabolized by that form will be eliminated more quickly than might have been predicted. This can result in inadequate drug levels, which can lead to serious problems if the condition being treated is not controlled. On the other hand, equally serious consequences can occur if the levels of an isozyme are decreased, as the rate of metabolism of a drug metabolized by that form might now be diminished to a point that produces toxic levels of a drug. The loss of drug metabolism can be particularly dangerous if a drug with a narrow therapeutic index is involved, due to the fact that toxic levels can be achieved rapidly. The risk of such a problem occurring is elevated in individuals who are taking many drugs at the same time, as the drugs can alter each other's metabolism.

1.3 THE IMMUNE SYSTEM AND CYTOCHROME P-450

One such situation in which cytochrome P-450 dependent metabolism is impaired occurs following the activation of host defense mechanisms (Renton et al, 1994). This decrease can have serious consequences, particularly if one is dealing with a drug with a narrow therapeutic index and serious toxic effects. A better understanding of the processes involved in this decrease in cytochrome P-450 dependent metabolism could

potentially lead to the prediction, or even prevention of this decrease in metabolism. A further potential benefit to understanding the processes involved is the possibility of intentionally altering selected P-450 forms. This might be useful in cases of drugs which are very expensive, rare, or so rapidly metabolized that they require frequent administration. If one could selectively decrease the metabolism of such a drug, it could be given in lower doses, and the frequency of the dose could be reduced. The cause of the alteration in cytochrome P-450 following exposure to infection, or activation of the immune system is not completely understood, but it is likely that the release of cytokines and other mediators is involved. During this process, monocytes, or macrophages, as they are called when they are activated and have migrated into the extravascular space, exert a protective function. Within the liver, the monocytes are fixed to the endothelial cells and are called Kupffer cells. They function to phagocytose, to present antigens, and to produce and release cytokines (Bouewns et al, 1992). Cytokines are low molecular weight (<80 kDa) peptides that are produced by many different cell types in response to a challenge. They act over a short spatial range, act at specific receptors, and act to control cell proliferation and differentiation processes. Some growth factors also fit these criteria, and are classified based on how they were first described (Whicher et al, 1990). Cytokines usually act locally to stimulate protective effects such as to increase vascular permeability, coagulation, bacterial killing and cell proliferation. They include the interleukins (IL-1 through IL-10), interferons (IFN alpha, beta, and gamma), tumor necrosis factors (TNF alpha and beta), platelet derived growth factor (PDGF), and transforming growth factor (TGF).

In 1978, it was first reported that there is an impairment of drug elimination during infectious disease in humans. Chang et al (1978) examined children who were taking theophylline, and who developed influenza A, influenza B, or adenovirus. It was observed that theophylline elimination is prolonged by about 68% in patients suffering from influenza compared with the rate of elimination one month after the infection (Chang et al, 1978). This decrease in elimination results in an increased risk of elevated levels of the drug. Tilorone, a small molecular weight interferon inducer, (Renton et al, 1976a and Leeson et al, 1976) causes a decrease in the levels of cytochrome P-450 and related metabolism. These observations are consistent with observations that substances which induce the formation of interferon in rats, including Mengo virus, tilorone, statalone, poly rI poly rC, LPS, liver RNA, and B. Pertussis vaccine, also lower cytochrome P-450 levels, and ethylmorphine N-demethylation (Renton et al, 1976b). Reports on the extent of the decrease in P-450 activity and content in response to interferon, or interferon inducers varies, and ranges from no reduction reported by some groups, to greater than 80% reduction by others (Williams, 1987; Kreamer et al, 1982). Renton proposed that influenza virus could induce interferon in humans, and lead to the decrease in P-450 levels, thus accounting for the altered elimination half life of theophylline that was reported in the influenza patients (Renton et al, 1978a). Such observations have not been restricted only to influenza and theophylline. It has now been reported by many groups that cytochrome P-450 dependent drug metabolism can be decreased following exposure to many infectious diseases, and situations in which the host defense system is activated.

Interferon was initially believed to be the mediator of the decrease in cytochrome

P-450 observed after administration of substances which would activate host defense mechanisms. Interferons play a role in protecting against viral infections. Substances known to induce interferon, most notably poly rI-rC, are able to depress cytochrome P-450 (Renton et al, 1976b). The profile of cytochrome P-450 depression correlates with elevated levels of serum interferon following the administration of encephalomyocarditis virus (Renton, 1981). In 1981, Singh et al exposed C_3H/HeJ mice to Newcastle Disease virus. This virus, which produces high levels of interferon, also depresses cytochrome P-450, but in C57BL/6J mice, which do not produce interferon in response to the virus, cytochrome P-450 was not effected (Singh et al, 1981). This data was viewed as strong evidence that IFN is the mediating factor in the depression of hepatic cytochrome P-450. This idea was confirmed following the direct administration of recombinant IFN, which was able to depress cytochrome P-450 (Singh et al, 1982). In this study it was also observed that purified recombinant IFN forms which lacked antiviral effects also were ineffective at depressing cytochrome P-450, suggesting that the cytochrome P-450 depressant, and antiviral effects of IFN were inseparable. IFN is believed to produce its antiviral effects through inhibition of viral protein synthesis by interfering with the viral protein translation (kinase activity), and by increasing the degradation of viral mRNA by activating an endonuclease (synthetase activity) (Baron et al, 1991). It may also affect host protein synthesis, and may depress cytochrome P-450 by inhibiting the synthesis of the apoprotein. The depression of cytochrome P-450 without depression of total protein within the hepatocyte may be explained by differences in turnover rates. The inhibition of protein synthesis is most obvious for proteins which have a high rate of synthesis. The

cytochrome P-450 forms that are most susceptible to interferon inducers are also those with relatively high turnover rates (el Azhary et al, 1979).

Despite the strong evidence for the involvement of IFN in the depression of cytochrome P-450, it has also been observed that some stimuli which produce the decrease in cytochrome P-450, do not induce IFN. Lipopolysaccharide (LPS), a constituent of the outer membrane of gram negative bacteria, is known to cause the activation of host defense mechanisms in a manner similar to septicemic shock. LPS is a potent depressant of cytochrome P-450, but is not a strong IFN inducer (Renton et al, 1976). Serum from animals treated with LPS, or culture medium from LPS treated peritoneal macrophage, when injected into naive animals, causes a decrease in P-450 levels, although there is no IFN detected in the culture medium (Williams, 1985). Administration of latex beads to animals produces a decrease in cytochrome P-450 levels which is mediated by Kupffer cells, which phagocytose the beads and become activated. This depression of cytochrome P-450 is not likely through interferon, as plasma from animals treated with latex bead do not have detectable interferon levels (Peterson et al, 1986). Other factors released from Kupffer cells are more likely to be involved, such as TNF, IL-1, and IL-6.

TNF, IL-1, and IL-6 have all been demonstrated to both individually, and in combination, depress multiple forms of cytochrome P-450 activity and expression in primary pig hepatocyte cultures (Monshouwer et al, 1996). In primary cultures of human hepatocytes, gene expression of CYP1A2, CYP2C, CYP2E1, and CYP3A was decreased after treatment with the inflammatory cytokines IL-1, IL-6, and TNF-α (Abdel-Razzak et

al, 1993). IFN, IL-1, IL-6, and TNF are all released in response to bacterial infection, and act as pyrogens. IL-1, IL-6, and TNF are considered to be multi functional cytokines and are part of the acute phase response. They have some overlap in function, in addition to their effect on cytochrome P-450. These cytokines are synthesized in response to various stimuli, and are then able to induce the expression of each other, and themselves, with the exception of IL-6, which does not induce the expression of IL-1 or TNF, but does induce its own expression (Akira et al, 1990). IL-2 has also been reported to depress cytochrome P-450 activity in high doses (Thal et al, 1994). This thesis is primarily interested in the effects of TNF as it depresses a broad range of cytochrome P-450 isoforms, and is an important mediator in toxic shock and sepsis in addition to its role in bacterial infection, LPS stimulation, and inflammation. Recombinant TNF administered intravenously to mice was able to produce a decrease in P-450 levels and in cytochrome P-450 dependent activity (Ghezzi et al, 1986). There are two identified TNF receptors. TNF receptor activation results in changes in phosphorylation of a number of proteins and can lead to cytotoxicity, antiviral activity, proliferation, and activation of the transcription factors NF-κB and Activator Protein-1 (AP-1) (fig. 3). The mechanism by which TNF and the other cytokines depress cytochrome P-450 remains unkown.

1.4 PROTEIN SYNTHESIS

When recombinant IFN was administered to mice in the presence of Actinomycin D or Puromycin, the decrease in cytochrome P-450 was prevented, indicating that an intermediate protein, produced in response to the interferon, is required for the subsequent

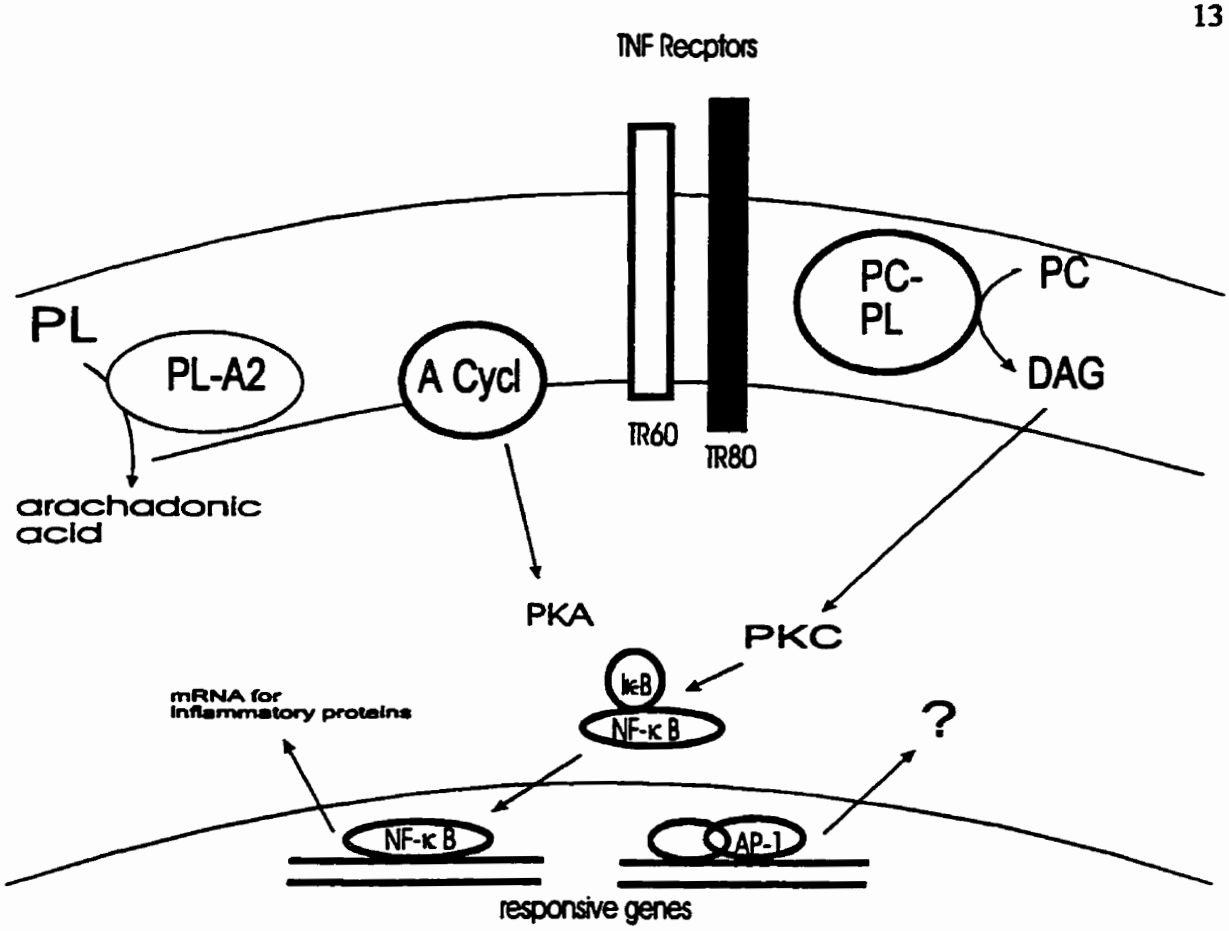


FIGURE 3

Activation of TNF Receptors
Activation of TR60 and/or TR80 leads to the production of arachadonic acid, and the activation of protein kinases, including protein kinase A (PKA) and protein kinase C (PKC). The activation of these kinases leads to transcriptional changes, which are due in part to the activation of the transcription factor NF-κB and AP-1. PL: phospholipids, PL-A_2: phospholipase A_2, DAG: diacylglycerol, PKA: protein kinase A, PKC: protein kinase C, PC: phosphtidylcholine (adapted from Pfizenmaier, 1992).

depression of cytochrome P-450 (Renton et al, 1990). Although there were many possibilities proposed for what could mediate this response, including xanthine oxidase (Ghezzi et al, 1984 and Moochala et al, 1991), other cytokines such as IL-6 (Williams et al, 1991 Chen et al, 1992), heme oxygenase (Fakuda et al, 1994) and nitric oxide synthase (Khatsenkoet al, 1993), the identity of this protein remains unknown. Of the above, heme oxygenase and nitric oxide synthase are the only ones which remain probable candidates (Cribb et al, 1993, Wright et al 1991) . Nitric oxide synthase has been proposed as a mediator for a variety of cytokines and cytochrome P-450 isoforms, but as will be discussed, there is considerable disagreement on the importance of nitric oxide synthase in this process, and the existence of an as yet unidentified protein mediator is still a real possibility. This thesis is directed at a) evaluating primary hepatocytes as a model in which to look for this protein, b) characterizing the depression of the specific isoform CYP1A in response to cytokines, c) attempting to inhibit this depression with selected inhibitors.

Although the synthesis of an intermediate protein is required to produce the depression in cytochrome P-450, there is an overall depression in hepatic protein synthesis in response to cytokine stimulation. The rate of degradation of proteins, indirectly identified as being apocytochromes P-450 based on their molecular weight, is actually decreased by 10% in hamsters treated for 24 hours with interferon, despite a 31% decrease in total cytochrome P-450 content. This is found to be due to a decrease in the rate of synthesis of these proteins (Moochhala et al, 1986). Using an oligonucleotide probe for CYP4A, it was found that the decrease in cytochrome P-450 protein following

IFN stimulation is due to a decrease in the amount of the mRNA coding for the apocytochrome (Renton et al, 1990). It has been determined that this decrease in the amount of mRNA for the apocytochrome is due in part to a decrease in their transcription, but also in part, due to an increase in the degradation of the mRNA for these apocytochromes (Delaporte et al, 1997).

1.5 NITRIC OXIDE

One of the main candidates for the identity of the intermediate protein is the inducible form of the enzyme nitric oxide synthase. It has received a great deal of attention recently, and yet, controversy still exists concerning the role that nitric oxide plays in the depression of cytochrome P-450 in response to cytokines. Nitric oxide is a soluble gas produced by many cell types. It has a wide range of effects, both inter- and intra- cellularly (Curran et al, 1991). It is involved in the control of blood pressure, neurotransmission, platelet aggregation, and the cytotoxic and cytostatic effects of macrophage (Ogden et al, 1991). Nitric oxide is derived from L-arginine and oxygen in a reaction catalyzed by an enzyme of the nitric oxide synthase group. Nitric oxide synthase exists in at least three forms; neuronal constitutive NOS (nNOS or NOS1), and endothelial constitutive NOS (eNOS or NOS3), which require calcium to bind calmodulin in order to become activated, produce small amounts of nitric oxide. A third form, which was called inducible NOS (iNOS), now called NOS2, produces large quantities of nitric oxide. In general, NOS2 must be synthesized in response to stimuli, but it has been found

constitutively in some resting epithelial cells. The distinguishing characteristic of this form is that it is not dependent on calcium for its activation, as the enzyme is able to bind calmodulin in the absence of calcium. Nitric oxide can activate soluble guanylate cyclase, can inhibit protein kinase C, can activate tyrosine kinase, can inactivate NF-κB, and can activate G-proteins (Kim et al, 1996).

In hepatocytes, no constitutive nitric oxide synthase has been identified, however, the inducible form has been identified, and can be induced by cytokines. Cocktails of cytokines (TNF, IL-1 and IFN) induce NOS2 (Geller et al, 1994) however there are reports of individual cytokines capable of inducing NOS2 as well. NOS2 is induced during the acute phase response, but it is regulated differently from acute phase proteins, and therefore is not strictly considered to be part of the acute phase response (Geller et al, 1994). Geller found that IL-1 and TNF are each able to induce NOS2 in rat hepatocytes. IL-6 is not able to induce NOS2 on its own, but the addition of IL-6 to the other cytokines increases the amount of nitric oxide produced (Geller et al, 1994).

Nitric oxide, like carbon monoxide, can bind to the heme iron of hemoproteins such as cytochrome P-450. Khatsenko and Wink (Khatsenko et al, 1993, and Wink et al, 1993) have shown that the addition of nitric oxide can impair cytochrome P-450 in microsomes. Khatsenko also demonstrated that the CYP2B1 activity was inversely related to the amount of nitric oxide produced from the nitric oxide generator, SIN-1. More interesting than this was that LPS stimulation of animals resulted in a) suppression of CYP2B1/2 activity, and b) elevation in plasma nitrate. Addition of the nitric oxide synthase inhibitor L-NAME, suppressed both the elevation of nitrate levels and the

depression in CYP2B1/2 activity, indicating a link between the formation of NO, and the depression of cytochrome P-450

Calson et al (1996) reported that NO is important in the depression of cytochrome P-450 (CYP1A2, CYP2B, CYP2C11, and CYP3A2) by IL-1 and by TNF, but not by IL-6. This study examined nitric oxide formation, cytochrome P-450 protein levels, a nitric oxide synthase inhibitor (L-NMA), and a nitric oxide donor (DETE/NONOate) (Carlson et al, 1996). IL-1 and TNF increase NO production, and produce a depression in cytochrome P-450. This depression is prevented by the addition of a nitric oxide synthase inhibitor. IL-6 produces a depression in cytochrome P-450 protein levels, but does not produce nitric oxide, and is not blocked by the addition of the nitric oxide synthase inhibitor. The nitric oxide donor is able to depress the cytochrome P-450 protein levels. The proposed mechanism for this decrease in protein involves NO binding to the heme moiety of cytochrome P-450 enzymes and destabilizing them, leading to an increase in degradation of the apocytochrome or the heme portion of the enzyme. As for the effects on mRNA, Stadler has reported that the nitric oxide synthase inhibitor L-NMA, is able to prevent the suppression of the CYP1A1 mRNA by cytokines (Stadler et al, 1994), suggesting that NO is important in the cytokine mediated decrease in CYP1A mRNA.

On the other hand, there is evidence that nitric oxide may not be involved. In a study using pig hepatocytes, it was found that four different cytokines (IL-1, IL-6, TNF, and IFN), as well as a cocktail of TNF, IL-1 and IFN are able to depress CYP3A enzyme activity by 12 hours, and the activity remained depressed at 24 hours. Neither IL-1 or TNF produce detectable amounts of NO as determined by nitrate and nitrite formation,

but the cocktail of IL-1, TNF and IFN is able to produce NO. The addition of the nitric oxide synthase inhibitor, L-NAME completely inhibits the production of NO, however, it is unable to prevent IL-1, TNF, or the cocktail from depressing the activity of CYP3A. These findings do not support a role for NO in the depression of this form of cytochrome P-450 (Monshouwer et al, 1996). Sewer et al (1997) reported that the depression of the constitutive CYP2C11 by IL-1 and LPS is independent of nitric oxide. The nitric oxide synthase inhibitor L-NMA was able to block nitric oxide production, but was unable to inhibit the effect of these stimuli on CYP2C11 mRNA or protein.

Additionally, the findings of Moochhala et al (1986) that the rate of degradation of cytochrome P-450 apoprotein is actually decreased following exposure to interferon is not consistent with a theory that proposes nitric oxide binding destabilizes the apoprotein.

Due to this conflicting information, there needs to be further investigation into the role of nitric oxide in cytokine mediated depression of cytochrome P-450. There have been many reports that link nitric oxide and the transcription factor NF-κB, and the involvement of a transcription factor in the synthesis of an unknown intermediate protein is possible. The importance that this transcription factor may have in the depression of cytochrome P-450 by cytokines has received little or no attention, however several lines of evidence point to a possible involvement.

1.6 NF-κB

It is proposed here that one of the factors which may be mediating the depression of cytochrome P-450 in response to cytokines is the transcription factor, nuclear factor kappa B, or NF-κB. NF-κB is actually made up of homo- and hetero dimers or trimers of two or three sub units from the Rel family that bind together. There are numerous subunits that can combine in various ways to form an NF-κB type transcription factor, but the combination which is most commonly referred to by the term NF-κB is the one made up of two subunits called p65 (Rel A) and p50 (NF-κB1). Different combinations of the various subunits may contribute to different regulatory effects, and could possibly be a means of providing specificity of response (Barnes et al, 1997). NF-κB was originally detected in B-lymphocytes, but has since been identified in many different cell types, and as mentioned, in many forms. They participate in gene activation related to defense mechanisms, and to cell proliferation processes. In most cells under normal conditions, NF-κB exists in an inactive form, located in the cytoplasm where it is bound to the inhibitor protein I–κB (fig. 4). I-κB also exists in several forms, again allowing for differential response, depending on the specific form present. NF-κB can be activated by many stimuli including endotoxin (LPS), cytokines (such as TNFα, IL-1, IL-2), UV light, viruses (including Influenza), and oxidants (such as peroxide, superoxide, and ozone). The pathway by which NF-κB becomes activated involves the generation of mitochondrial oxidants, followed by phosphorylation and ubiquination of the inhibitor protein I-κB. This

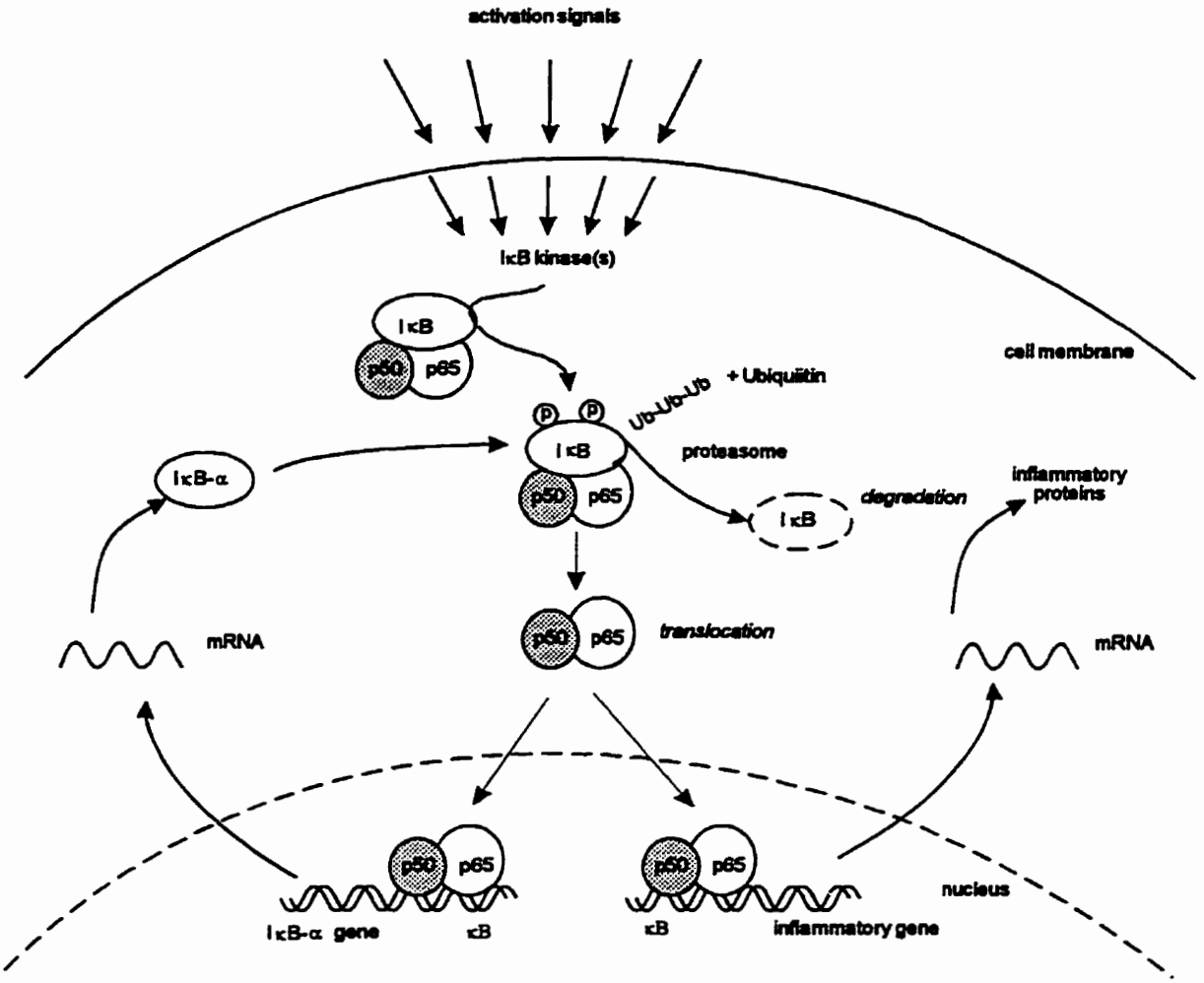


FIGURE 4

Activation of the transcription Factor NF-κB
Stimuli such as IL-1, TNF, hydrogen peroxide, superoxide, and Influenza virus can activate NF-κB. This transcription factor then triggers the production of the inhibitory protein I-κB, as well as inflammatory proteins such as IL-6, NOS2, and ICAM-1. (adapted from Barnes, 1997)

targets the inhibitory protein for degradation by a proteosome, leaving the active NF-κB composed of the two sub units p50 and p65. The active NF-κB migrates to the nucleus, where it binds to genes with κB promoter sequences, (GGGACTTTCC) (Barnes et al, 1997). This binding results in an increased transcription of the genes containing this sequence. Among the target genes are surface immunoglobulin, adhesion molecules, cytokines, acute phase response genes, and others. The gene encoding the form of the inhibitory protein I-κBα also contains the κB promoter sequence, therefore NF-κB activation increases the transcription of this inhibitor, and thus turns itself off. The gene for the form I-κBβ does not contain the sequence, and thus cells in which this form is dominant, NF-κB activation is likely to be longer lasting (Barnes et al, 1997).

In the hepatocyte, three forms of the inhibitory protein are present, I-κBα, I-κBβ, and I-κBγ. Stimulation of the human hepablastoma cell line HepG2 with TNF results in a biphasic response. Within 15 minutes, I-κBα and I-κBβ disappear, and NF-κB translocates to the nucleus. Within 60 minutes, the I-κB level is double the control, and nuclear binding of NF-κB is at control levels. By 120 minutes, the I-κBα level returns to control values, but I-κBβ is still depressed, and the nuclear binding of NF-κB is increased again, and remains increased for at least 360 minutes (Han et al, 1997).

In many conditions in which cytochrome P-450 is depressed, NF-κB is also activated. Specific targets for NF-κB within the hepatocyte include adhesion molecule genes such as intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule (VCAM), or E selectin, acute phase protein genes such as human serum amyloid A, rat

angiotensinogen, mouse complement factor B (Freedman et al, 1992), as well as granulocyte- macrophage colony stimulating factor (GM-CSF) (Newton et al, 1996) and the enzyme, inducible nitric oxide synthase (NOS2).

The possible involvement of NOS2 in the cytokine mediated depression of cytochrome P-450 has already been discussed. NOS2 is a target for NF-κB, as well as for AP-1, and for the TNF responsive element (Diaz-Guerra et al, 1996). The activation of NF-κB is thought to be an absolute requirement for the expression of NOS2 in response to endotoxin and to IFN (Nathan et al, 1994 and Xie et al, 1994). Therefore, if the involvement of nitric oxide is required for the depression of cytochrome P-450, this suggests that the involvement of the transcription factor NF-κB may also be a requirement.

Within the liver, NF-κB has been identified in hepatocytes and Kupffer cells. Freedman *et al* prepared protein extracts of cytoplasmic and nuclear fractions of Kupffer cells and hepatocytes, and showed that Kupffer cells, like macrophages, contain constitutively active, nuclear bound NF-κB. In rats exposed for 24 hours to LPS, a stimulant of the acute phase response, there is no increase in the nuclear binding activity of the transcription factor in the Kupffer cells. In hepatocytes, little active NF-κB is found in the control cells, however, there is an increase in the nuclear binding of NF-κB in hepatocytes from rats exposed to LPS for 24 hours. The administration of LPS to rats results in a depression of cytochrome P-450. Hepatocytes placed in primary culture for 3 hours express more active NF-κB than isolated cells prior to culture (Freedman et al, 1992). Plating and culture of hepatocytes also results in a rapid decrease in the levels of

cytochrome P-450 (Renton et al, 1978).

The addition of IL-6 to primary hepatocytes did not increase the nuclear binding activity of NF-κB (Freedman et al, 1992). This could perhaps be because it was already fully activated, but it is not discounted that IL-6 is simply not a stimulant of NF-κB. IL-6, as mentioned is able to depress cytochrome P-450, but it does not induce nitric oxide. This inability to activate NF-κB may be a clue to this difference between IL-6 and the other inflammatory cytokines.

Oxidative stress is another example of a situation in which there is both an increase in the activation of NF-kB, and a depression of cytochrome P-450. The activation of NF-κB is a response to oxidative stress, and has been demonstrated to be activated in cultured hepatocytes after treatment with hydrogen peroxide. Oxidative stress mimics many of the effects of inflammation. In a study using hydrogen peroxide as a source of oxidative stress, there is a reduction in the accumulation of CYP1A1 and CYP1A2 mRNA in primary rat hepatocytes (Barker, 1994). This observed depression in mRNA is consistent with the response of cytochrome P-450 to cytokines.

NF-κB has been identified as a potential component of hepatic acute phase response. NF-κB activation has been shown to occur in hepatocytes, Kupffer cells, and liver endothelial cells of animals treated with the endotoxin *Salmonella enteritidis* for 1 and 5 hours (Essani et al, 1996). IP injection of 5 μg of TNF into rats causes a strong induction of NF-κB 30 minutes after injection. The same effect of TNF on NF-κB is seen in liver cell culture experiments. (Fausto et al, 1995). IL-1 and TNF are reported to be the most potent cytokines at inducing NF-κB (DiDonato et al, 1997). Again, endotoxin,

TNF, and IL-1 are all able to produce the depression of cytochrome P-450. All of these findings suggest that NF-κB may be important in the response of hepatocytes to inflammatory stimuli. Whether there may be a link between NF-κB activation and the depression in cytochrome P-450 seen in response to cytokines is a topic of this thesis.

1.7 OBJECTIVES

The underlying hypothesis that directed this thesis is that TNF, and possibly other cytokines, depress cytochrome P-450 through a mechanism involving the activation of the transcription factor, NF-κB. It is proposed that this transcription factor then signals the production of protein, among which are those involved in the depression of cytochrome P-450. Included in the proteins believed to be involved, is the enzyme NOS2, although other, as yet undetermined proteins are also proposed to be involved (fig. 5). This thesis aims to examine this process of cytokine mediated depression of CYP1A activity, and to determine the ability of inhibitors at the level of protein synthesis within the hepatocyte, at the level of the production of nitric oxide, and at the level of the transcription factor, NF-κB.

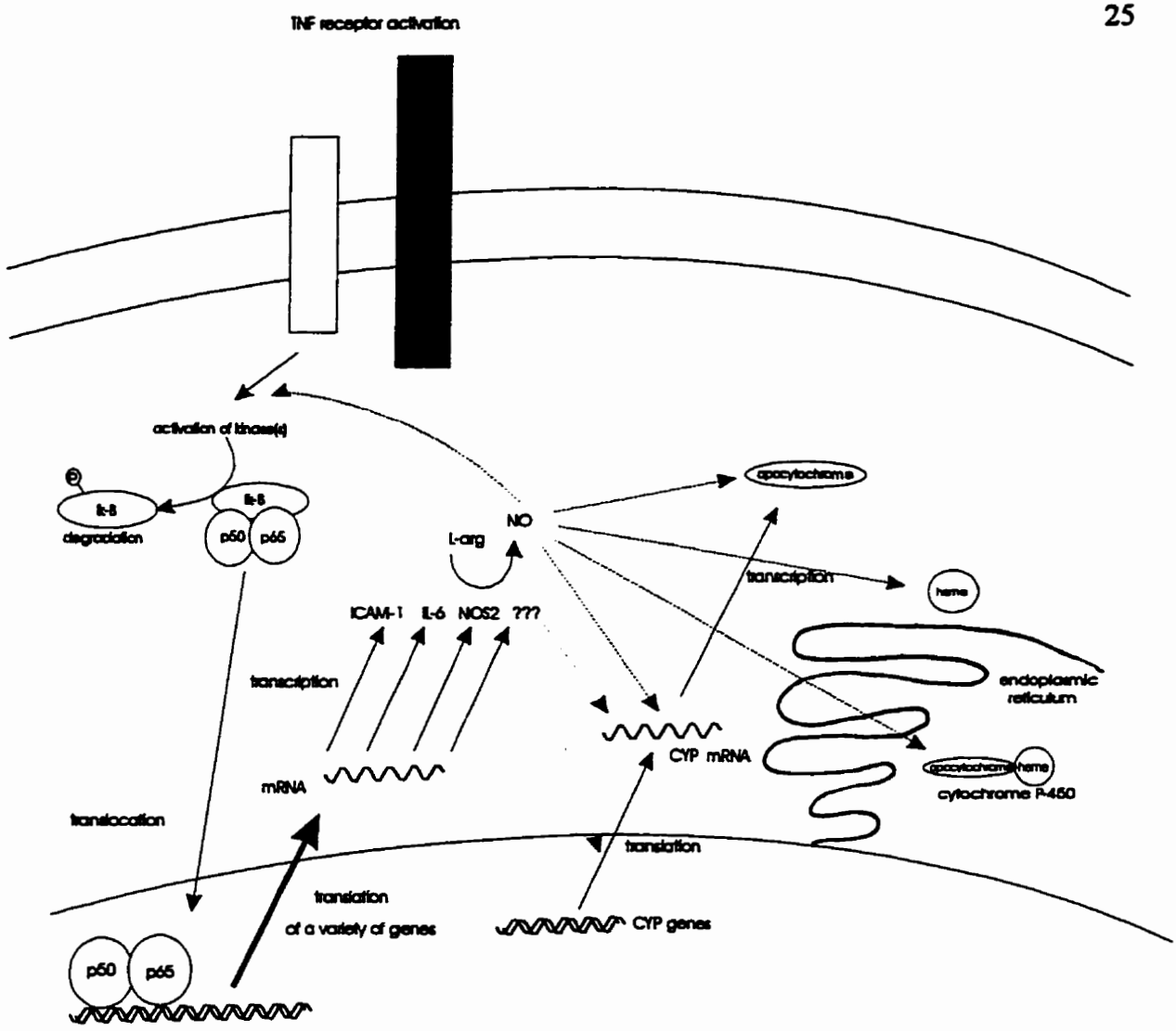


FIGURE 5

Hypothesis

TNF receptor activation leads to the activation of the transcription factor NF- KB. This leads to an increase in the production of a variety of proteins including ICAM-1, IL-6, NOS2, and other as yet undetermined proteins. These may effect CYP1A activity by increasing the degradation of the apocytochrome or heme groups, by functionally blocking the enzyme, by increasing the degradation of, or decreasing the production of CYP mRNA.

2.0 MATERIALS and METHODS

2.1 MATERIALS

BDH

ammonium persulfate

glycerol

Phenol-Folin-Ciocalteau Reagent

Boenringer Manheim

Actinomycin D

acrylamide

bis acrylamide

Fisher Scientific

methanol

Gentest

anti goat IgG alkaline phosphatase conjugate

Anti-rat CYP1A1 polyclonal

Gibco

antibiotic-antimycotic

fetal bovine serum (FBS)

MEM-α

RPMI 1640

tumor necrosis factor (TNF-α)

Molecular Probes

Fluorecent Latex Beads

Sigma Chemical Company

beta Naphthoflavone (BNF)

bovine serum albumin (BSA)

bromophenol blue

deoxynivalenol (DON)

di benzanthracene (DBA)

dimethyl sulfoxide (DMSO)

dimethylformamide

ethylenediamine tetraacetic acid (EDTA)

ethoxyresorufin

Fluorescent Latex Beads

glycine

L-imino ornithine (NIO)

L-N6-(1-imino ethyl) lysine (NIL)

lipopolysaccharide (LPS)

methanol

N,N,N',N'-tetramethylethylenediamine (TEMED)

N-benzoyl-tyrosine ethyl ester (BTEE)

N-nitro arginine (NNA)

N-tosyl-l-phenylalanine chloromethyl ketone (TPCK)

nicotinamdie adenine dinucleotide phosphate (reduced from) (NADPH)

nitro blue tetrazolium (NBT)

nitrocellulose paper

polyoxyethyylenesorbitan monolaurate (Tween 20)

puromycin

resorufin

sodium dithionate

sodium dodecyl sulfate

tris[hydromethyl] aminomethane hydrochloride (tris HCL)

Trypan Blue

Type IV collagenase

2.2 ANIMALS

Male CFW mice, approximately 25 g in weight, were held on a clay chip bedding in the Carleton Animal Care facility. They were allowed to acclimatize to their environment for at least 5 days, but not more that 8 days before use.

2.3 CYP1A INDUCTION BY BNF

When BNF was used as the inducing agent, CYP1A was induced *in vivo* prior to isolation of the cells. Mice were administered 140 μg/Kg BNF suspended in olive oil, as a 0.2 ml IP injection, once daily for the three days preceding cell isolation.

2.4 CELL ISOLATION

2.4.1 Perfusion Technique

This procedure was performed as described previously (Renton, 1978b). Calcium free buffer (4.8 g HEPES, 16.6 g NaCl, and 1 g KCl, in 2 L distilled water, pH = 7.4 with NaOH) and a buffer containing Type IV collagenase (as above with sufficient collagenase Type IV to give 70 units of digestive activity per ml buffer (approximately 0.15 - 0.2 mg/ml)) were placed in an ice bath. Size 14 Tygon tubing led from the cold buffers to a two way valve. The tubing passed through a pump to a condenser coil. The condenser coil was attached to a water bath maintained at 37°C. This allowed the buffer to be warmed before passing into a canula made of 15 gauge tubing (fig. 6).

Animals were anesthetized with approximately 0.4 mg sodium pentobarbital per

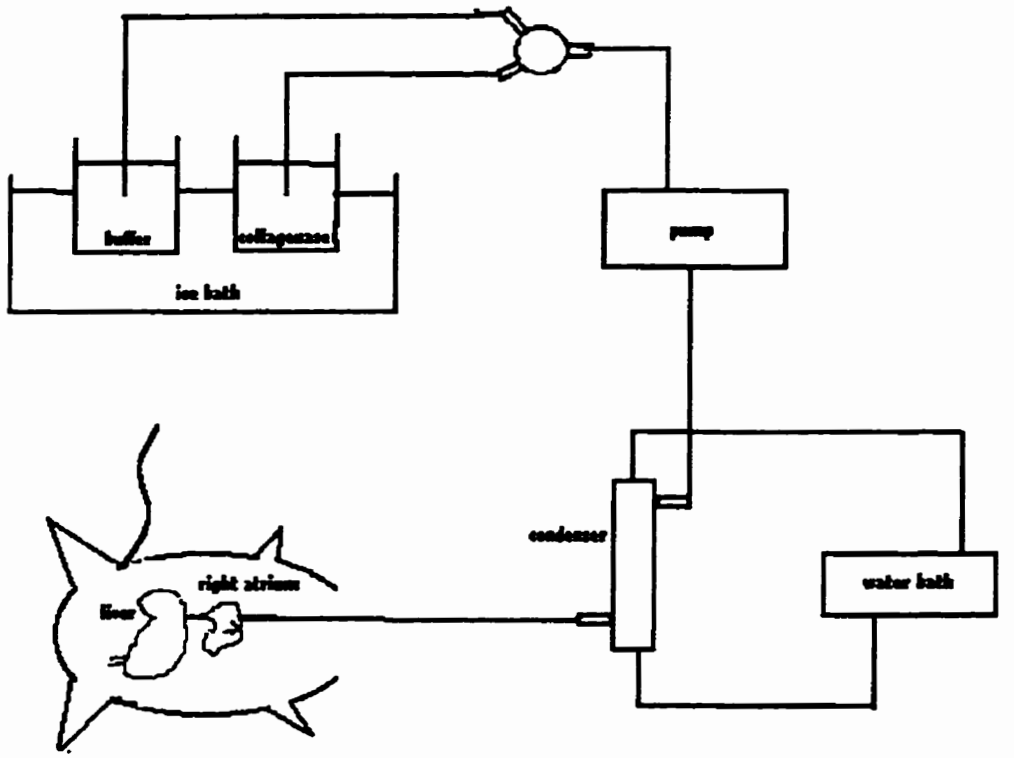


FIGURE 6

Method for isolating mouse hepatocytes
Buffer or collagenase buffer is warmed in a condenser coil before perfusing the liver via the right atrium (adapted from Renton, 1987).

gram body weight. The abdominal cavity was opened, and the contents were shifted out of the body to the left, in order to locate the portal vein. The chest cavity was opened, to expose the heart, and a small incision was made in the right atrium. Using the 15 gauge tygon tubing, the inferior vena cava was cannulated through this incision. Calcium free buffer, warmed to 37°C, was pumped through the cannula at a flow rate of 0.5 ml/min. The portal vein was then cut, and the flow rate was increased to 10 ml/min for 2 minutes. Successful cannulation resulted in a flushing of blood from the liver turning it from deep red to light brown. Also, the flow of buffer could be observed in the abdominal cavity. After 2 minutes, the buffer was switched to the buffer containing type IV collagenase. This buffer was also warmed to 37°C, and the flow rate was held at 10 ml/min for 10 minutes to allow for the digestion of the collagen in the liver. During this time the liver became glossy in appearance, and leaky. The perfusate was then switched back to calcium free buffer for a final 2 minutes, to rinse the collagenase from the liver.

The liver was carefully removed and placed into a 9 cm diameter petri dish, with 35 ml serum free RPMI. It was gently scraped with a blunt dissection tool, to dissociate the cells. The suspension was passed through a layer of gauze to remove large pieces of debris, and collected in a 50 ml sterile tube. This was placed on ice until the isolation procedure was completed for all the mice being used in the given experiment.

2.4.2 Cell Fractionation

Once cells were isolated from all of the required mice, the tubes were inverted several times to resuspend the cells. They were placed on ice for 20-40 minutes during which time, most of the hepatocytes settle to the bottom of the tube. The supernatant containing the Kupffer cells, was placed in a separate tube, and also kept on ice. The loose hepatocyte pellet was resuspended in 35 ml cold calcium free buffer. To purify the hepatocyte fraction, it was centrifuged at 50 x G for 1 minute, the supernatant discarded and resuspended in cold buffer. This was repeated until the cells had been centrifuged three times. After the final centrifugation, the pellet was suspended in 10 ml warm RPMI with 10% fetal bovine serum and antibiotic-antimycotic. The cells from all mice were then pooled into one tube, or flask, (depending on the total volume), and the cells were counted. Viability of the hepatocytes was determined by Trypan Blue exclusion. This procedure generally yielded a cell viability above 80%, and a viable cell count of approximately 5 x 10^6 cells/ml. The hepatocytes were then plated at a cell density of approximately 0.5-1 x 10^6 cells/ml in a 5 ml plate in MEM-α with 5% Fetal Bovine Serum (FBS) or RPMI 1640 with 10 % FBS, antibiotic-antimycotic, and where indicated, DBA. All plates in a given experiment received an equal volume of the cell suspension, so that within a given experiment, the plates were homogenous. The separation of Kupffer cells was achieved by an initial low speed (50 x G) centrifuge step for 1 min, where the resulting hepatocyte pellet was discarded, followed by a higher speed (500 x G) centrifuge step for 3 minutes, resulting in a small pellet, which contained the Kupffer cells. This pellet was resuspended in 5 ml warm RPMI 1640 with 10% FBS, and antibiotic. The cells

from all required mice were pooled, and examined under a microscope to confirm the presence of small cells. They were then plated in a total volume of 5 ml RPMI 1640 with 10% FBS, and antibiotic-antimycotic. Again, the plates received an equal volume of the cell suspension, so that within a given experiment, all plates were homogenous. All cells were allowed to attach overnight, and the next day, fresh RPMI-1640 with 10% FBS, and antibiotic-antimycotic was added.

2.4.3 Cell Identification

For cell counts at the time of plating, hepatocytes were identified qualitatively based on their size, shape and overall appearance. Kupffer cells were not counted prior to plating, as they could not be adequately identified. After plating, Kupffer cells were identified by their ability to phagocytose fluorescent latex beads. Fluorescent latex beads (2-3 x 10^5 beads/µl) were added to plates of Kupffer cells and hepatocytes. Cells which had incorporated these beads after 3 hours at 37^{0}C, were identified as Kupffer cells. These were visualized, and photographed under a fluorescent microscope. This was used to confirm that Kupffer cells were present, and more importantly, was used to ensure that the hepatocyte preparations were relatively free from contaminating Kupffer cells. Slides were prepared of both Kupffer cell and hepatocyte fractions exposed to latex beads.

2.4.4 Slide Preparation

For the slide preparation, cells were plated on cover slips. After allowing sufficient incubation time to adhere, fresh medium was added (as described in section 2.4). The

latex beads were then added for the indicated times and conditions. The cover slips were rinsed in PBS (16 g NaCl, 0.4 g KCl, 2.88 g Na_2HPO_4, 0.48 g KH_2PO_4, and water to 2 L pH = 7.4), fixed with methanol at -20°C for 5 minutes, rinsed again in PBS. A slide was prepared by placing a drop of mounting solution (1:1 glycerol:PBS) on a glass slide and inverting the cover slips onto this drop.

2.5 CELL TREATMENT

2.5.1 CYP1A Induction with DBA

Generally, hepatocytes were induced to express increased levels of CYP1A with 50 nM Dibenzanthracene (DBA), prepared in dimethyl sulfoxide (DMSO). DMSO on its own may alter CYP1A activity, but because it was added to both treated and control, this effect would be accounted for. This induction was achieved by adding DBA at the time of cell isolation, so that the levels of P-450 were elevated at the time of stimulation, then stopped at the time of stimulation to assess effects of treatment on the rate at which P-450 decreases.

2.5.2 Preparation of Conditioned Medium

Multiple plates of Kupffer cells were stimulated for 24 hours with 50, or 100 µg/ml of lipopolysaccharide (LPS), added as 50 µl of a concentrated solution made up in sterile, distilled water. Control Kupffer cells received 50 µl of water. The medium was collected and pooled, following the 24 hr stimulation period. This medium was referred to

as Kupffer cell conditioned medium (KCM) with and without LPS, (KCM-LPS, and KCM-H_2O, respectively). This was then used to stimulate hepatocytes.

Deoxynivalenol conditioned medium was prepared in a similar manner. Kupffer cells were stimulated with 75 μg/ml of deoxynivalenol (DON) for 24 hours. The medium was collected and pooled. This medium (KCM-DON) was used to stimulate hepatocytes.

2.5.3 Hepatocyte Treatment

For the conditioned medium experiments, the medium was removed from the hepatocytes, and replaced with 4 ml of Kupffer cell conditioned medium, and 1 ml fresh RPMI 1640 with 10% FBS, in order to ensure sufficient nutrients were present. Fresh antibiotic-antimycotic was also added at this time. This mixture was then incubated with the hepatocytes for the time indicated.

For the experiments where TNF was used, the medium on the induced hepatocytes was changed as described in section 2.5. The indicated cells also received 10 ng/ml TNF, added as 50 μl of a concentrated solution prepared in medium. (The TNF was dissolved in PBS, then diluted with RPMI 1640 containing 0.1% BSA. 50 μl of 0.1% BSA added on its own did not effect the activity of CYP1A, as measured by EROD (data not shown)). All other drugs were added in similar manner (i.e. as 50 μl of a concentrated solution, with controls receiving 50 μl of vehicle when the vehicle was not medium). The final concentrations and the length of stimulation are indicated in the results section. When inhibitors were added, they were added prior to TNF, or KCM. All cells were incubated at 37°C in 5% CO_2. All cell treatment and handling was carried out using sterile

techniques, in a containment hood.

2.6 ASSAYS ON PRIMARY CULTURED HEPATOCYTES

2.6.1 Ethoxy resorufin-O-deethylase (EROD) Assay for Cultured Hepatocytes

The activity of CYP1A was determined using a modification of the method described by Burke (1977). This assay (Paton, 1996) was chosen because of its ease, its reproducibility, and its compatibility with plated hepatocytes, as opposed to microsomes, which many cytochrome P-450 assays require. CYP1A converts ethoxyresorufin to the fluorescent product resorufin. The activity of the enzyme is expressed as the amount of resorufin, determined using a fluorimeter, produced in a given amount of time per milligram of total protein. A stock solution of ethoxy resorufin was prepared in 10% DMSO, 90% 0.1 M KH_2PO_4 to a final concentration of 100 μM ethoxyresorufin, and 30 μl of this was added to each 5 ml plate. This was allowed to incubate at 37°C, at 5% CO_2 for 30 minutes. Following the incubation, 2.5 ml of the medium was removed and placed in a test tube. The plates were returned to the incubator. The fluorescence was measured using a Perkin-Elmer Fluorescence Spectrophotometer and with an excitation wavelength of 510 nm and an emission wavelength of 586 nm. The amount of fluorescence of a known concentration of resorufin (50 nM) was measured, and using this as a standard, the amount of resorufin in the tubes was determined.

The activity of the enzyme CYP1A in the plated hepatocytes was expressed as picomoles of resorufin produced by one milligram of total protein in one minute (pmol/mg/min).

2.6.2 Protein Determination

Medium was removed from the culture plates and cells were rinsed with PBS. Following this, 2 ml of PBS was added to the plates, and they were scraped with a rubber policeman. The suspension of cells was collected and sonicated for 3 minutes to disrupt the membranes.

The determination of the protein content of each plate was conducted as described by Lowry (1951). A solution (mix #1) was prepared containing 98% of 2% sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH), 1% of 1% Copper Sulfate ($CuSO_4 \cdot H_2O$), and 1% of 2% Sodium Potassium Tartrate ($NaKC_4H_4O_6 \cdot H_2O$) in advance. The disrupted cells were thoroughly vortexed, then diluted with PBS 50- 100 µl of sample to 200 -150 µl of PBS to give a total volume of 250 µl. For the standard, 1 mg/ml BSA, and for the blank, water were diluted 100 µl standard or water to 150 µl PBS. 1.25 ml of mix #1 was added to each tube. The tubes were left for 10 min. Then 0.125 ml of 2 N Phenol Folin-Ciocalteau reagent, diluted 1:1 with water, was added to each tube. Each tube was vortexed, and allowed to incubate at room temperature for 30 min. The spectrophotometer was calibrated using the blank solution. Protein was detected by a change in absorbance at 700 nm, using 0.061 mg/ml BSA as a protein standard.

2.6.3 Western Immunoblot Analysis

Cells from a treatment group within an experiment were pooled together, and concentrated by centrifugation. The protein was determined as described in section 2.5.2. Samples were diluted in PBS in order that 10 µl gives 40 µg of protein. These were then further diluted 1:1 with Laemmli buffer (489 mg Tris HCL, 5 ml glycerol, 5 mg Bromophenol blue, 5 ml of 10% SDS and water to make 25 ml). These were boiled for 3 minutes along with a low range molecular weight marker.

An agarose gel was prepared by boiling 10 mg/ml agarose in water for 5 minutes. The gel apparatus was set in this to create a seal on the bottom. Running gel {4.5 ml stock acrylamide (29 g acrylamide, 1 g bisacrylamide, and 70 ml water), 4.5 ml 4 X running gel (16.95 g Tris base, 4 ml of 10% SDS, and water to make 100 ml. PH = 8.8), 90 µl of 100 mg/ml ammonium persulfate, 9 ml water, and 24 µl TEMED} was then layered onto the set agarose. Stacking gel {0.75 ml stock acrylamide, 3 ml water, 3.75 ml 2 X stacking gel (3 g Tris base, 2 ml of 10% SDS, water to 100 m, pH = 6.8), 75 µl of 100 mg/ml ammonium persulfate, 15 µl TEMED} was layered onto the running gel, and a comb was placed into the apparatus to create sample wells. Once set, the comb was removed, as was the agarose gel that had been used to create the seal. The apparatus was set into the tray, and the apparatus was filled with electrode buffer (33 ml of 10 X electrode buffer (7.56 g of Tris base, 36 g of glycine, 25 ml SDS, water to 250 ml, pH = 8.3) and water to 330 ml). 10 µl of sample or molecular weight marker were placed per well. The apparatus was set to run at 75 volts for 20 minutes, to allow the samples to

stack. The voltage was then increased to 125 volts for about an hour to allow the gel to run. The gel was removed, and the distance moved of the molecular weight markers and samples was recorded

A stack of blotting paper, gel, nitrocellulose paper, and more blotting paper was set on the electrophoresis apparatus with the papers soaked in transfer buffer (3.03 g Tris HCL, 11.25 g Glycine, 200 ml Methanol, 5 ml of 10%SDS, water to 1 L pH = 8.3).The gel was allowed to transfer for 1.5 hours at 300 mA. The nitrocellulose was then soaked in BLOTTO buffer (10 g Carnation low fat milk, 4 g Bovine serum albumin, 200 ml water) for 1 hour with rocking. The nitrocellulose paper was then left overnight at 4^0C in primary antibody buffer {40 µl polyclonal anti-rat CYP1A in 10 ml dilution buffer (20 ml BLOTTO and 10 µl Tween 20)}. The membrane was washed 3 times for 10 minutes each wash in washing solution {300 ml PBS (16 g NaCl, 0.4 g KCl, 2.88 g Na_2HPO_4, 0.48 g KH_2PO_4, and water to 2 L pH = 7.4) and 150 µl Tween 20}. The membrane was then incubated for 1 hour with 20 µl of anti-goat IgG (alkaline phosphatase conjugate) in 50 ml of antibody dilution buffer. The membrane was washed 3 times for 10 minutes per wash in the washing solution.

To develop the bands, the membrane was equilibrated in alkaline phosphatase buffer (7.9 g Tris HCL, 2.9 g NaCl, 5.0 g MgCl, and water to 500 ml, pH = 9.0) then exposed to alkaline phosphatase mix {10 ml alkaline phosphatase buffer, 132 l of (50 mg NBT per ml of 70% dimethylformamide) and 66 ml of (25 mg BCIP per ml dimethylformamide)}. When the bands became dark in color, the membranes were immersed in stop buffer (1.58 g Tris HCL, 0.93 g EDTA, and water to 500 ml, pH = 8.0).

The membranes were rinsed in water and air dried. The membranes were then photographed and analyzed using molecular analyst (Bio Rad) densitometry program.

2.7 ANIMAL TREATMENT FOR *iv vivo* STUDIES

2.7.1 Deoxynivalenol (DON)Treatment

Deoxynivalenol was diluted to 1 mg/ml in PBS adjusted to a pH of 9.44. Mice were administered 20 mg/Kg Deoxynivalenol by oral gavage, 24 hours prior to liver collection. Control animals received vehicle only. This dose and method was used as it was reported to produce an increase in mRNA for various cytokines.

2.7.2 N-tosyl-l-phenylalanine chloromethyl ketone (TPCK) Treatment

N-tosyl-l-phenylalanine chloromethyl ketone was diluted in 50% ethanol to give a final concentration of 6 mg/ml. Mice were given a 30 mg/Kg IP injection of TPCK 24 hours prior to liver collection. Control animals received vehicle only. This dose would give approximately 170 μM plasma concentration, assuming a 25 g mouse has a volume of distribution of 12.5 ml.

2.8 MICROSOMAL PREPARATION

Animals were sacrificed by cervical dislocation. The livers were removed and rinsed in cold 1.15% KCl, and weighed. The livers were then homogenized in 1.15% KCl with 10 strokes of the loose homogenizer, and 1 stroke with the tight homogenizer. This

homogenate was centrifuged in the Beckman J2-21 at 10 000 X G for 10 minutes at 4°C. The pellet of this spin would contain nuclei, plasma membrane sheets, mitochondria, lysosome, peroxisome, Golgi membranes, and some rough endoplasmic reticulum (Graham, 1984). The supernatant was collected and centrifuged in the Beckman L-60 Ultracentrifuge at 146 000 X G for 40 minutes at 4°C. The pellet of this spin contains microsomes of smooth and rough endoplasmic reticulum, Golgi, and the plasma membrane (mainly from the sinusoidal surface) (Graham, 1984). This pellet was then resuspended in 1 ml glycerol buffer per milligram of liver. The glycerol buffer consisted of 25% of 200 mM potassium phosphate (KH_2PO_4), 35% of 1.15 % Potassium Chloride (KCl), 20% glycerol, and 20% H_2O. This suspension was homogenized with 7 strokes in a tight homogenizer.

2.9 ASSAYS ON MICROSOMES

2.9.1 Ethoxy resorufin-O- deethylase (EROD) Assay for Microsomes

CYP1A activity was determined in the microsomes as described by Burke (1976). A solution of 2 ml of 0.1 M Potassium Phosphate (KH_2PO_4), 50 µl of undiluted microsomes, and 10 µl of 100 µM ethoxyresorufin was prepared in a cuvette made of special optical glass designed for use in the 320-2500 nm range. This solution was incubated in a 37°C water bath for 2 minutes. To this was added 10 µl of 25 mM NADPH prepared in 0.1 M KH_2PO_4, and a timer started immediately. The fluorescence was detected using a Perkin-Elmer Fluorescence Spectrophotometer and with an excitation

wavelength of 510 nm and an emission wavelength of 586 nm. The Florescence readings were recorded every minute for four minutes. These fluorescence values were plotted against time, and the slope was determined. A standard was prepared as above, with the omission of the ethoxyresorufin. Following the addition of NADPH, a baseline fluorescence was obtained, and 10 µl of 10 µM resorufin was added. The change in absorbance following the addition of the resorufin was recorded, and this was used to calculate the amount of resorufin, in picomoles produced per minute in the microsome samples.

2.9.2 Protein Determination

Protein was determined as described in section 2.5.2, with the exception of the dilution of samples and standards. Microsomal samples were diluted 1/400 with water, and 250 µl of this dilute microsome suspension was added to 1.25 ml of mix #1. A standard curve was prepared using 50, 100 and 200 µg/ml BSA, and the slope of this curve was used to calculate the concentrations of the samples.

2.9.3 Total Cytochrome P-450

Total cytochrome P-450 level was determined by the method of Omura (1964). Microsomes were diluted in 1 M KH_2PO_4, to give 4 ml of a final protein concentration of 1 mg/ml. This solution was placed in a cuvette, and a small amount of sodium dithionte was added. A baseline absorbance scan from 500 nm - 400 nm was obtained using the Beckman DU-70. The sample cuvette was then bubbled with carbon monoxide. The

difference spectrum was obtained. The difference between the baseline (490 nm) and the peak (450 nm) represents the amount of total cytochrome P-450 present. Using the extinction coefficient 91 OD/mM cytochrome P-450/cm, the amount of cytochrome P-450 in the sample can be determined. The total cytochrome P-450 content was expressed as nmol of cytochrome P-450 per mg of protein

2.10 Statistical Analysis

For the comparison of two groups, a students paired t-test was performed. In the case where more than two groups were compared, an ANOVA was performed, with a Newman-Keul post hoc analysis.

For cell cultured experiments in which one pool is presented, the ANOVA was performed on the raw data. For pooled experiments, the mean of the control was determined within an experiment. All data within an experiment, including the control values, were then multiplied by a constant, in order to normalize the data as a percent of the control. An ANOVA was then performed on the percent of control data, and a Newman-Keul post-hoc analysis was performed.

3.0 RESULTS

3.1 CELL SEPARATION

Hepatocytes were separated from Kupffer cells and other non-parenchymal cells by a series of centrifugation steps. Kupffer cells were then identified by their ability to phagocytose fluorescent beads, as illustrated in figures 7, 8 and 9. The Kupffer cell fraction (fig. 7) contained cells which were heavily labeled with the fluorescent beads following incubation for 3 hours at room temperature (20 ^{0}C). If these cells were incubated with latex beads for 3 hours at 4 ^{0}C (fig. 8), little uptake of these beads occurred. The hepatocyte fraction (fig. 9) had only a few, if any (98.3 ± 1.8 % free) cells containing the latex beads, indicating that there was little or no contamination of the parenchymal cell fraction with Kupffer cells. In all cases (fig. 7, 8 and 9), there were residual beads which failed to rinse off the plate with PBS, which are identified by their failure to correspond spatially with a cell.

3.2 INDUCTION OF CYP1A ACTIVITY

CYP1A is constitutively expressed in hepatocytes, and can be measured in hepatic microsomes prepared from untreated mice, but in simple primary hepatocyte cultures the activity is low, and this restricts the study of factors that cause a decrease in the enzyme activity or expression. In order to compensate for this decreased activity in cell culture, chemical inducers of the enzyme were used. This method was chosen over other methods

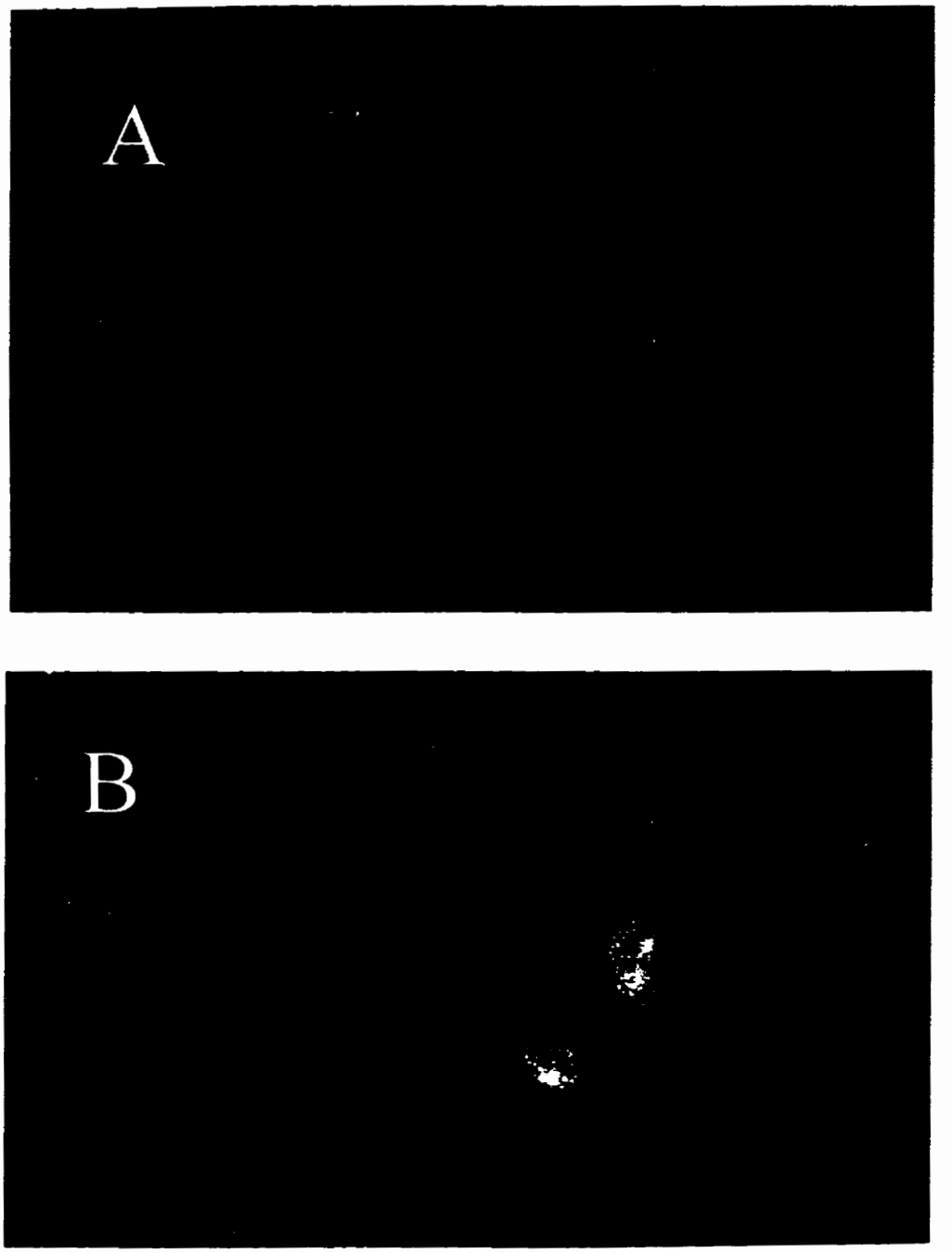


FIGURE 7
Kupffer Cells
Kupffer cell fraction exposed to latex beads at room temperature (20 ^{0}C) for 3 hours. Upper panel is photographed under phase contrast, lower panel is under fluorescence. Photos were taken at 40 X magnification.

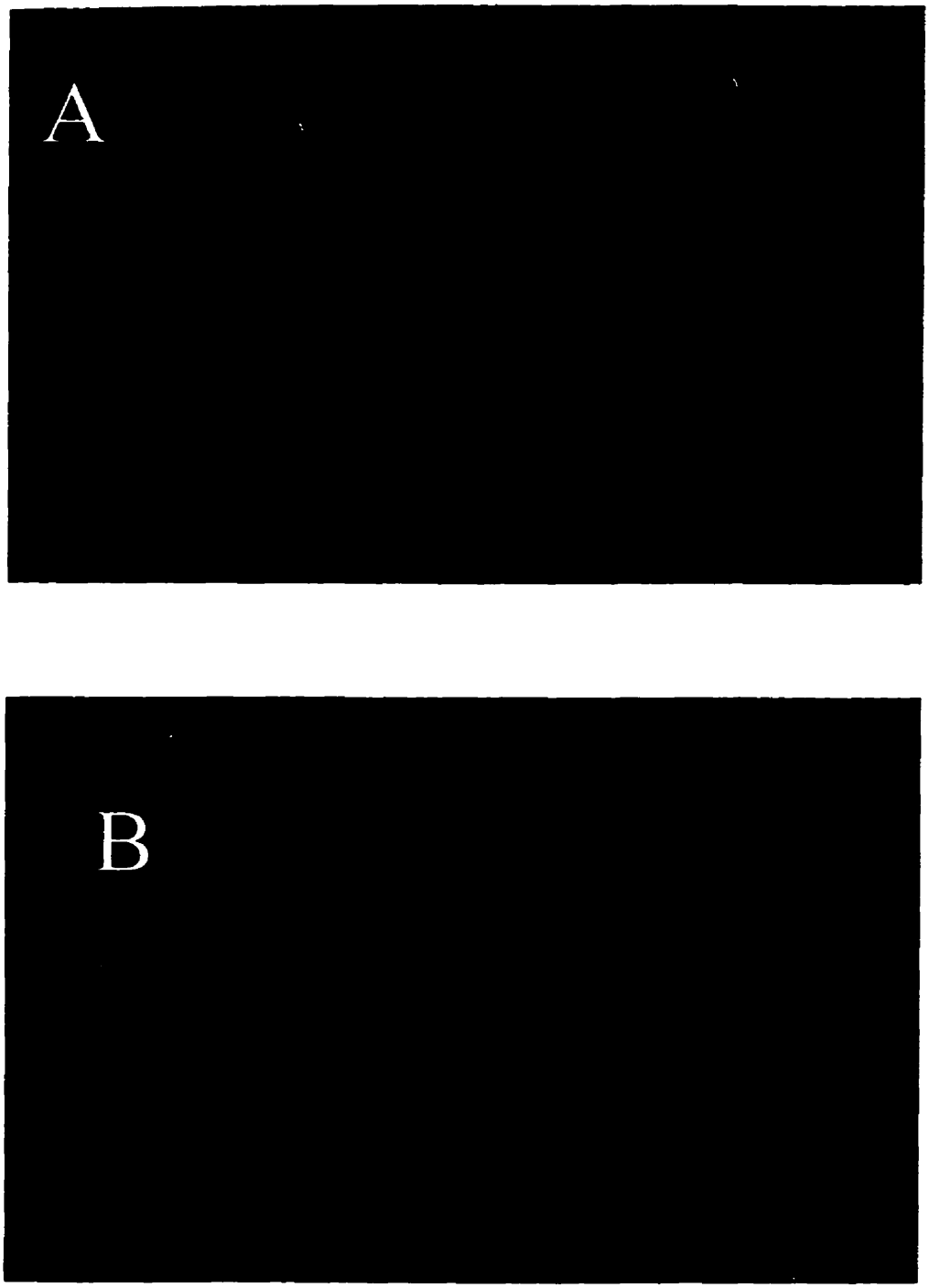


FIGURE 8
Kupffer Cells
Kupffer cell fraction exposed to latex beads at 4 ^{0}C for 3 hours. Upper panel is photographed under phase contrast, lower panel is under fluorescence. Photos were taken at 40 X magnification.

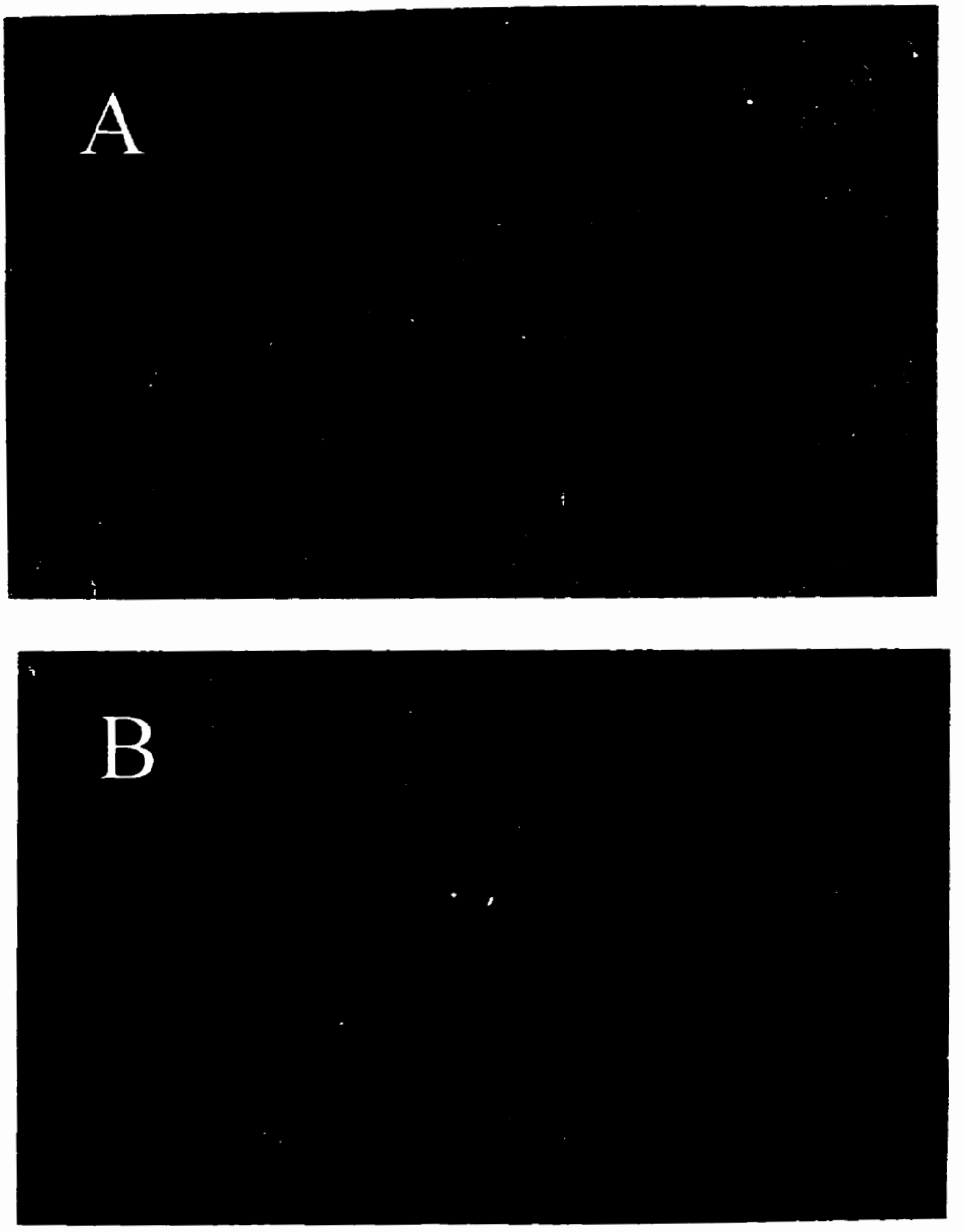


FIGURE 9
Hepatocytes
Hepatocyte fraction exposed to latex beads at room temperature (20 ^{0}C) for 3 hours. Upper panel is photographed under phase contrast, lower panel is under fluorescence. Photos were taken at 40 X magnification.

of maintaining elevated enzyme levels, such as 3 dimensional collagen matrices or rotating culture flasks, because of the simplicity, the cost effectiveness, and time efficiency.

3.2.1 *In Vivo* Induction of CYP1A

The CYP1A activity in the hepatocytes could be increased by pretreating the mice with an enzyme inducer. The treatment of the mice with BNF (140 μg/kg IP once daily for three days) (Delaporte, 1995) was effective at inducing hepatocytes such that the activity remained elevated after the cells were isolated as a primary culture (Fig. 10). The total time in culture for experiments in which BNF was used as the inducer ranged from 24 to 40 hours.

3.2.2 *In Vitro* Induction of CYP1A

The CYP1A activity could be also induced by the addition of an inducer directly to the culture plates following the isolation of the hepatocytes. The treatment of the cells with DBA (50 nM) (Paton, 1996) was effective at elevating activity of CYP1A in piated hepatocytes. The activity was elevated as early as 15 hours, and remained elevated relative to control (DMSO only) after 24 hours (Fig. 11). The total time in culture for hepatocytes in experiments where cells were induced in culture with DBA ranged from 40 to 60 hours.

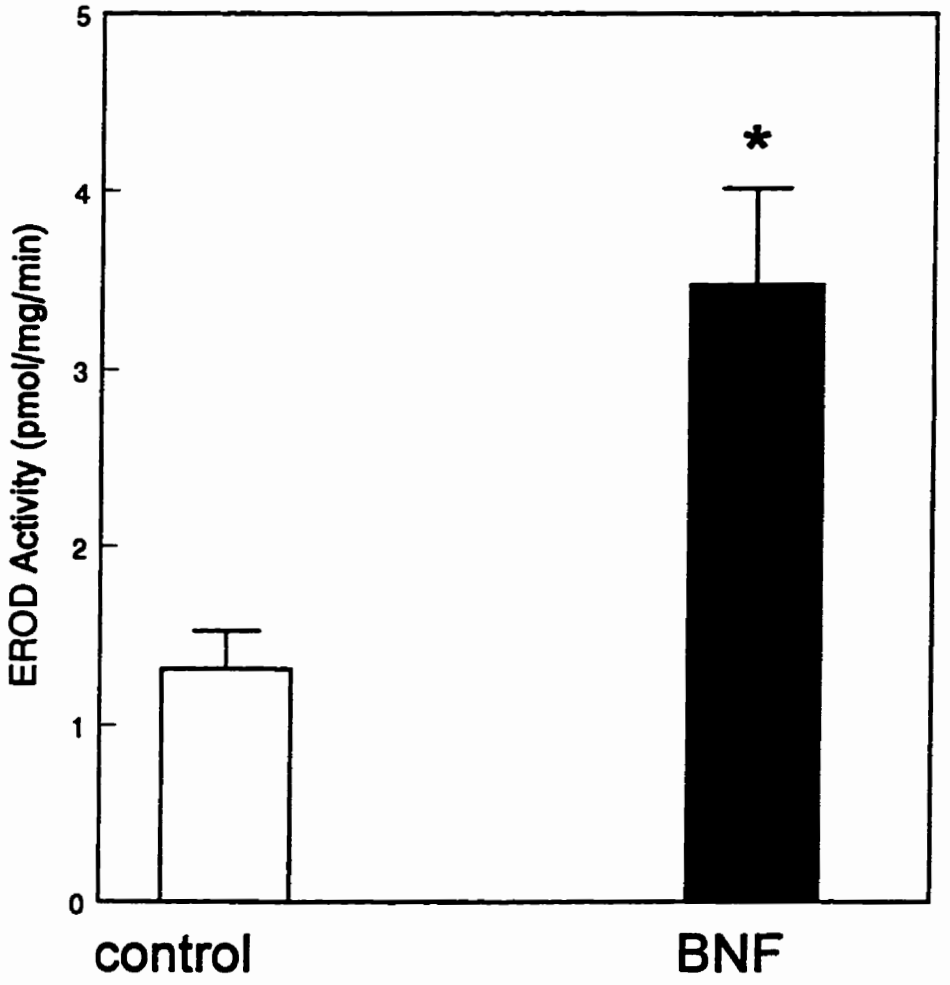


FIGURE 10

Induction of CYP1A *in vivo* with BNF
CYP1A was induced prior to hepatocyte isolation with 140 μmol/kg BNF by ip injection once per day for three days.
Hepatocytes were measured after 24 hours in culture.
n = 4 for each bar, using 1 homogeneous pool obtained from 4 to 8 mice.
*significant difference from control. $p < 0.05$.

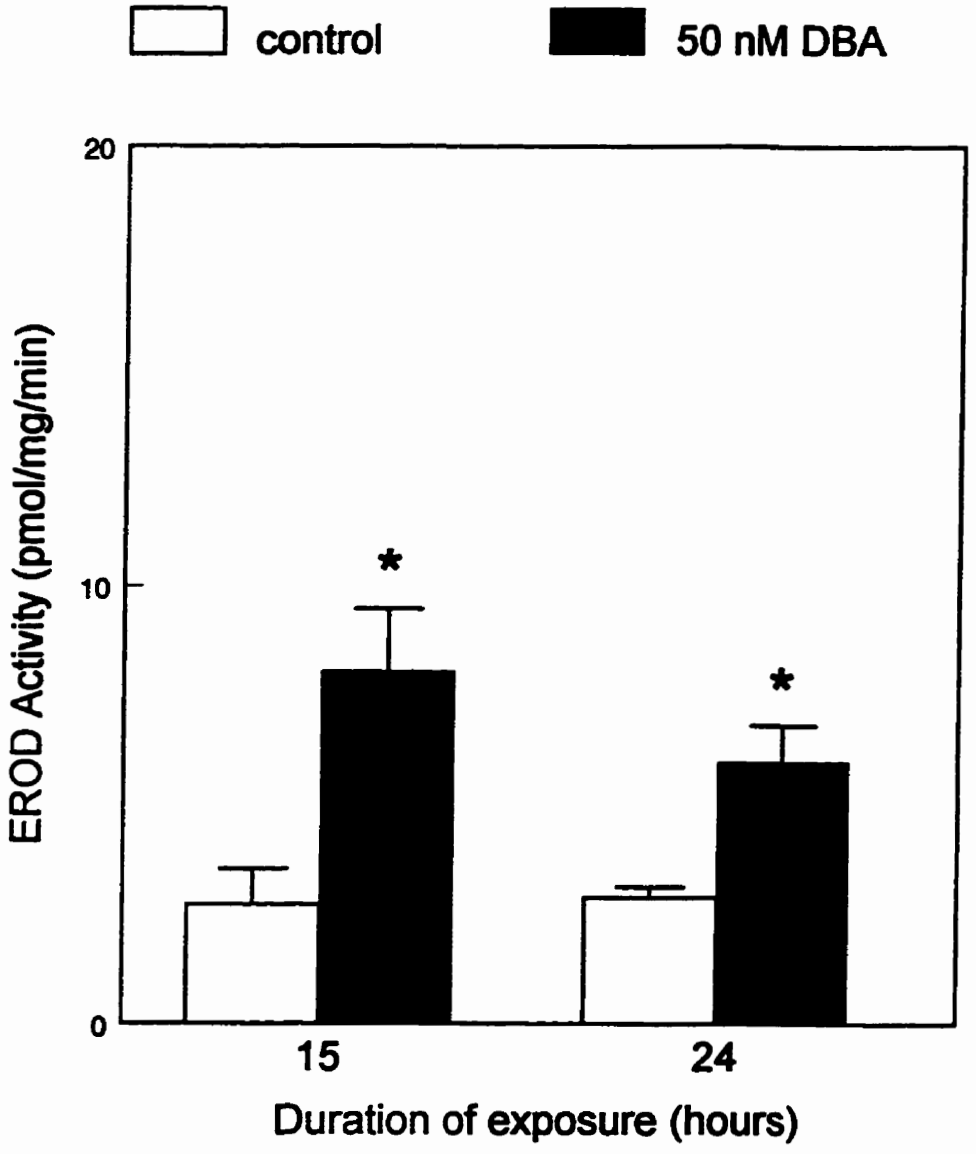


FIGURE 11

Induction of CYP1A *in vitro* with DBA
Cells which had been allowed time to attach (overnight) were exposed to DBA (50 nM) for the indicated time.
n = 3 for each bar, using 1 homogeneous pool obtained from 4 to 8 mice .
* significant difference from control values (adjacent open bar) $p < 0.05$.

3.3 CYTOKINES AND CYP1A

To confirm the suitability of this model for the study of the decrease in P-450 activity associated with activation of host defense, hepatocytes were exposed to immunoactive factors expected to depress CYP1A activity. It had previously been established that macrophages can be stimulated by lipopolysaccharide to produce components which cause a decrease in induced and/or constitutive CYP1A activity in isolated hepatocytes (Paton, 1995). In this study, primary culture of Kupffer cells, the resident monocytes of the liver, were exposed to LPS, and the conditioned medium from this was added to hepatocytes. Kupffer cells, like macrophages, produce components, including IL-1, IL-6, TNF, and NO, all of which are able to depress cytochrome P-450 activity and /or levels.

3.3.1 Kupffer Cell Conditioned Medium Produced by LPS

The ability of LPS to produce a depression in CYP1A directly, or via Kupffer cell conditioned medium was assessed, as illustrated in figures 12 to 16. Kupffer cell conditioned medium (KCM) was produced by removing the medium from cultures of Kupffer cells, which had been exposed to water or LPS (100μg/ml) for 24 hours. This Kupffer cell conditioned medium was supplemented with 20% fresh medium, as well as fresh antibiotic-antimycotic, and then added to the hepatocyte fraction. Figures 12, 13, 14, and 15 are single representative experiments. Figure 16 is the average of 7 (bars 1,2, and 3) or 5 (bar 4) experiments. Medium from Kupffer cells which had been exposed to LPS caused a decrease in CYP1A activity after 10 hours (fig. 12) and 24 (fig. 13, fig.

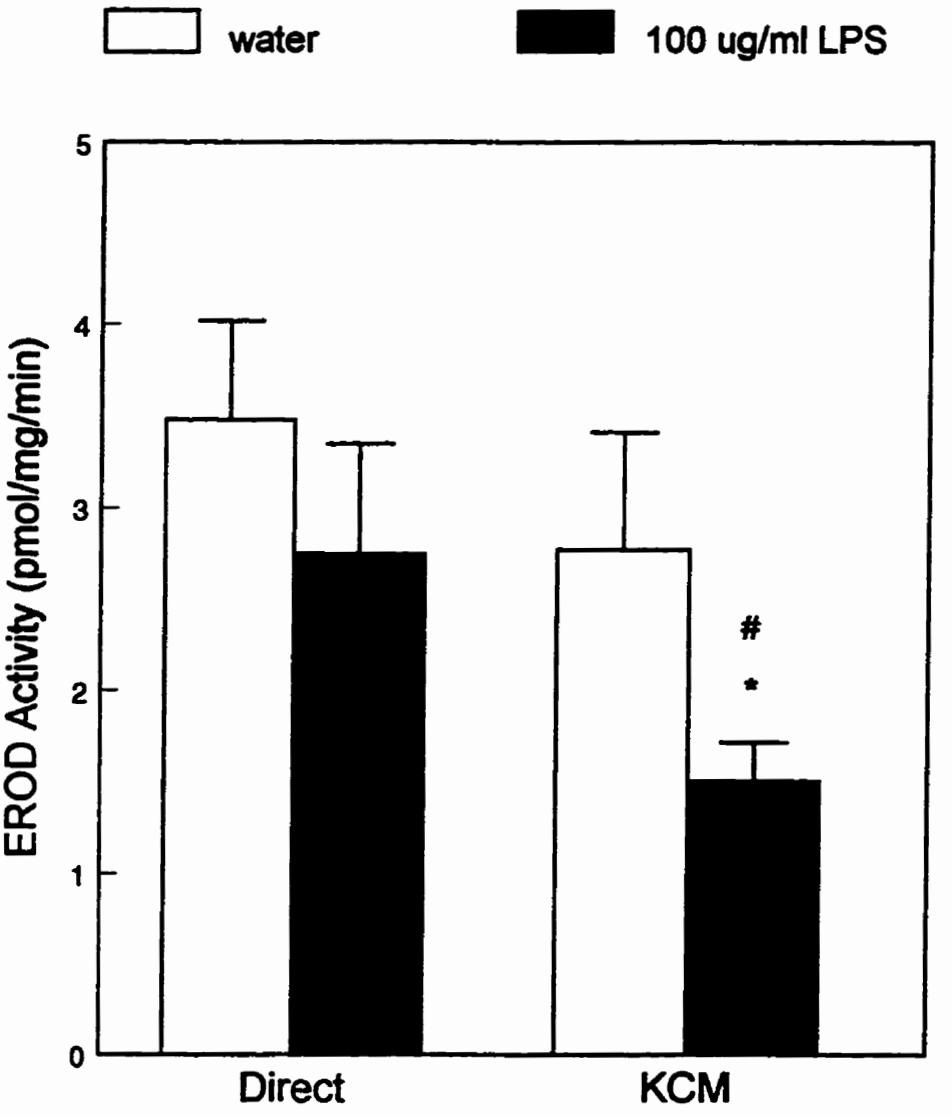


FIGURE 12

CYP1A activity in hepatocytes treated with LPS or KCM-LPS for 10 hours.
n = 4 using 1 homogeneous pool obtained from 4 to 8 mice.
* significant difference from control value (adjacent open bar). $p < 0.05$.
# significant difference from the untreated control cells (bar 1). $p < 0.05$.

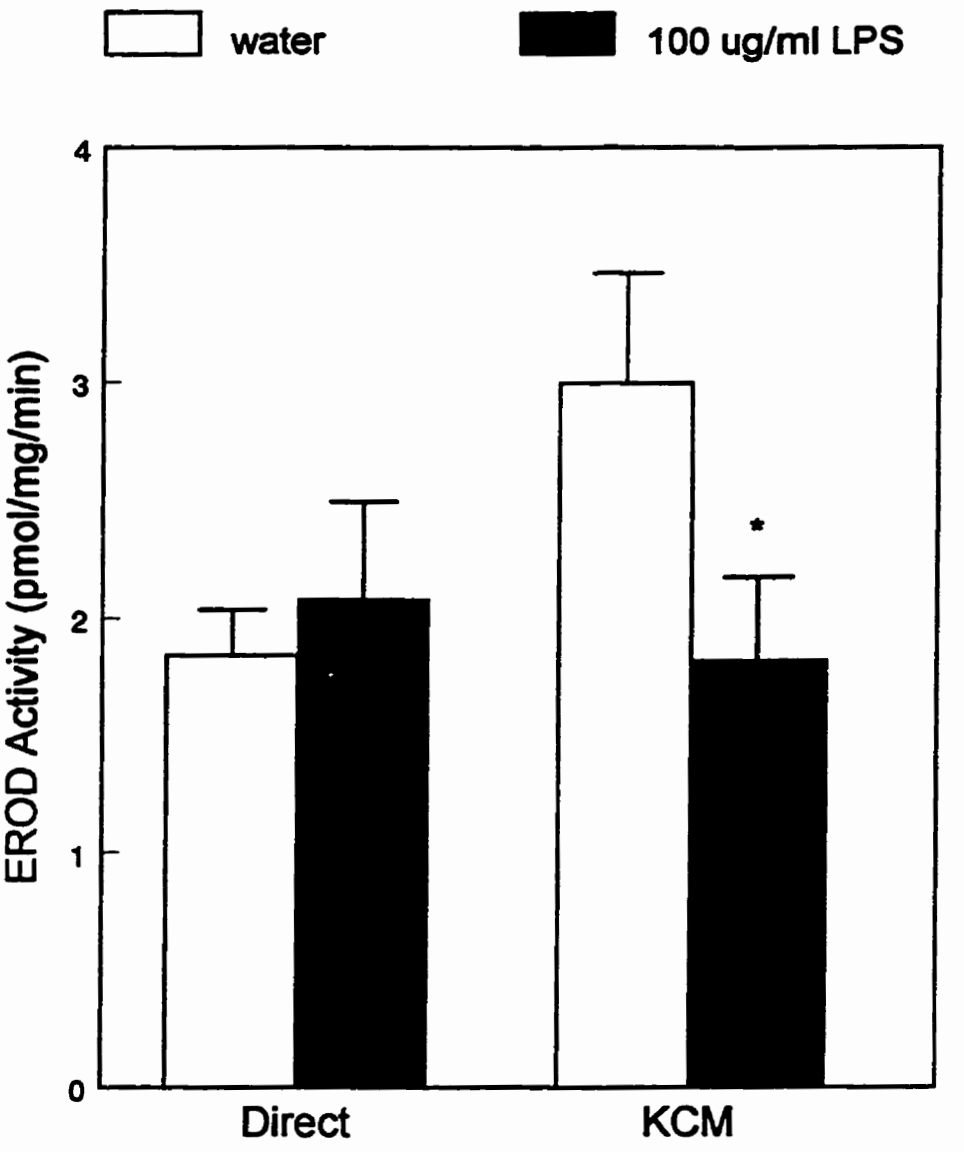


FIGURE 13

CYP1A activity in hepatocytes treated with LPS or KCM-LPS for 24 hours.
n = 4 using 1 homogeneous pool obtained from 4 to 8 mice.
* significant difference from control value (adjacent open bar). $p < 0.05$.

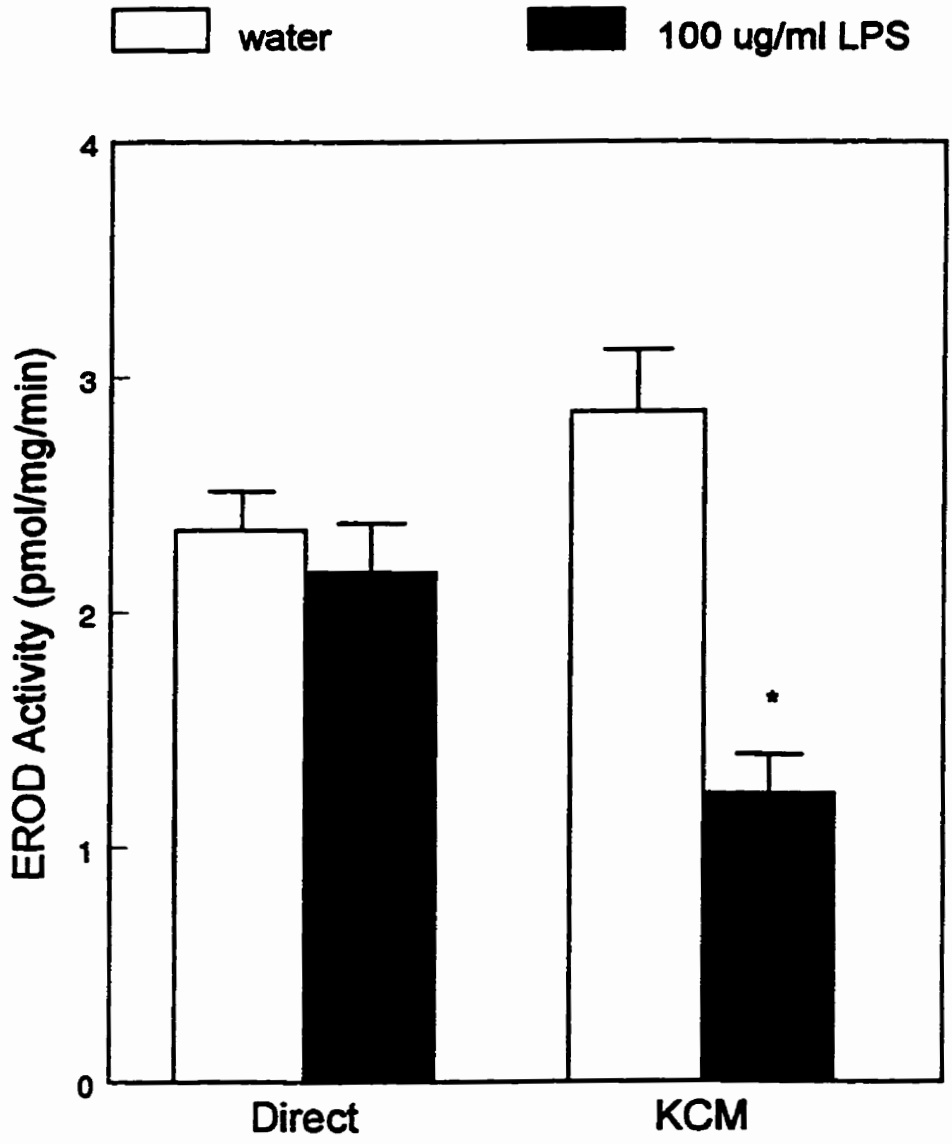


FIGURE 14

CYP1A activity in hepatocytes treated with LPS or filtered KCM-LPS for 10 hours
n = 4 using 1 homogeneous pool obtained from 4 to 8 mice.
* significant difference from control value (adjacent open bar). $p < 0.05$.

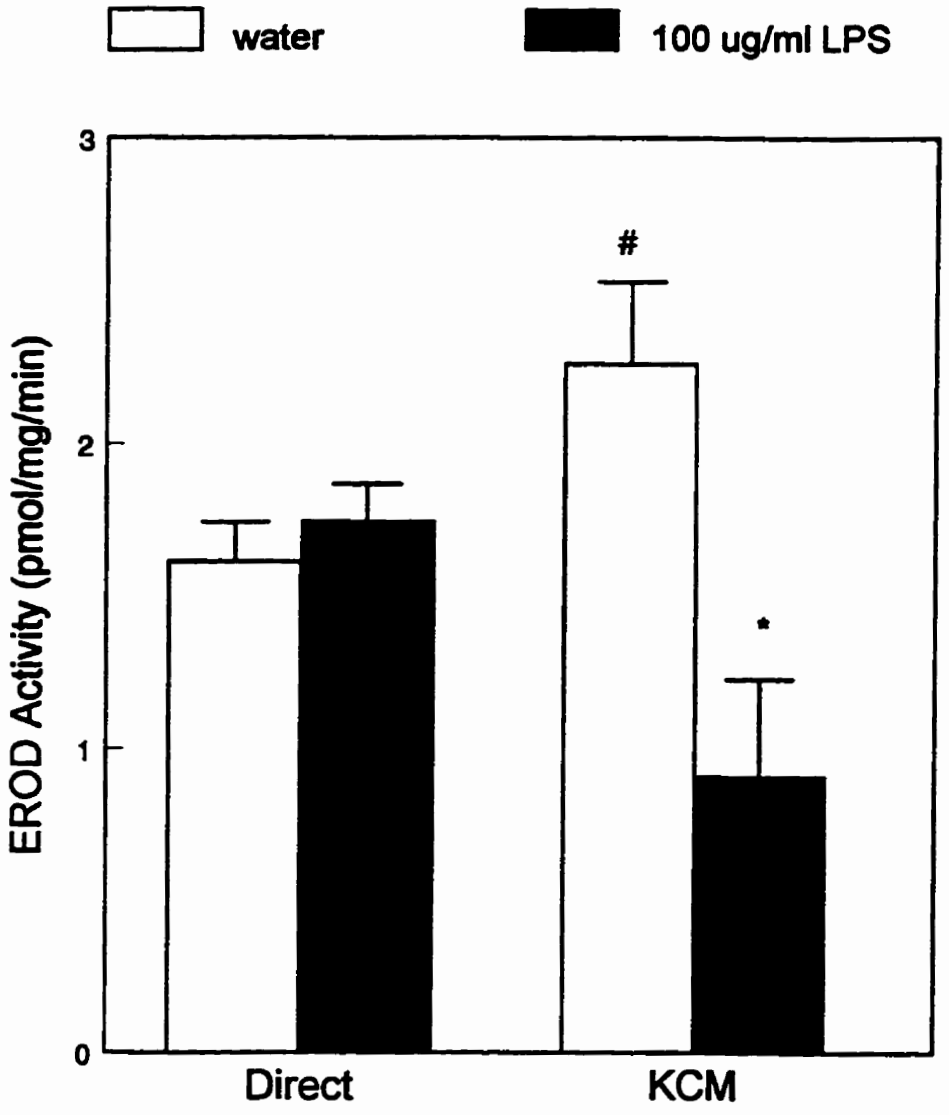


FIGURE 15

CYP1A activity in hepatocytes treated with LPS or filtered KCM-LPS for 20 hours

n = 4 using 1 homogeneous pool obtained from 4 to 8 mice.

* significant difference from control value (adjacent open bar). $p < 0.05$.

significant difference from untreated control cells (bar 1). $p < 0.05$.

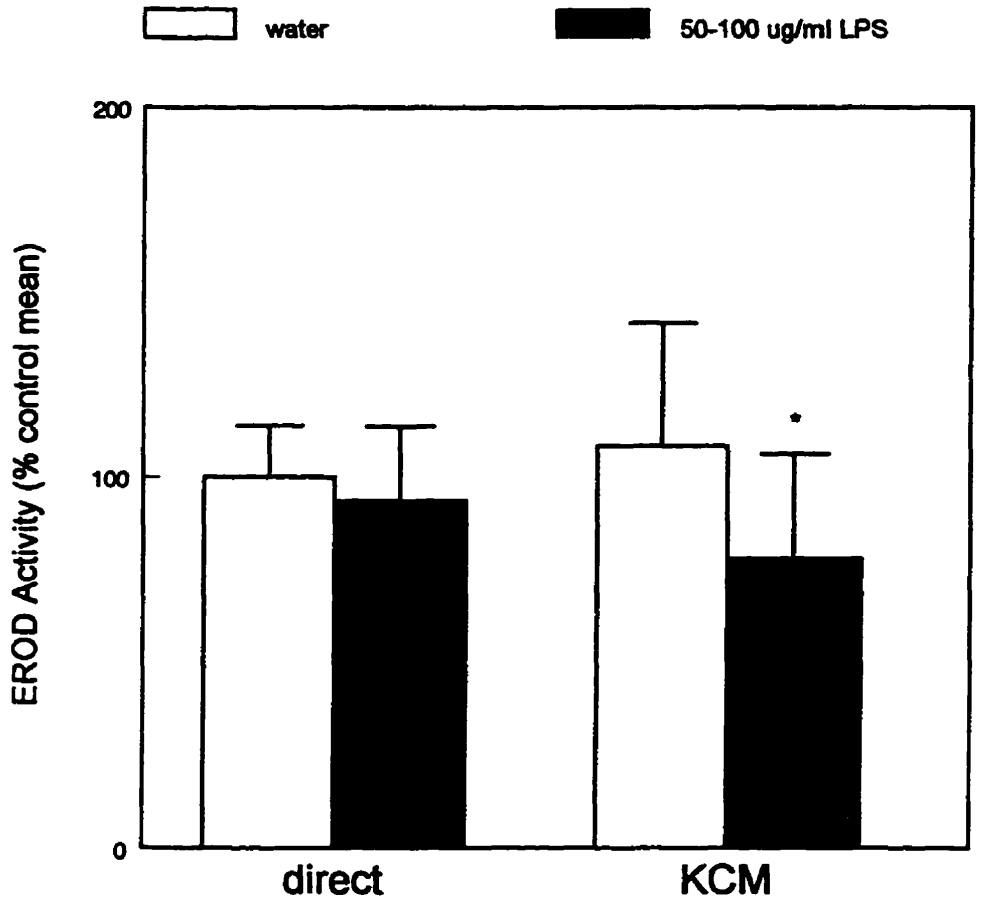


FIGURE 16

CYP1A activity in hepatocytes treated with LPS or KCM-LPS for 24 hours (pooled)

CYP1A was induced by administering BNF to mice prior to hepatocyte isolation.

DIRECT: LPS was added directly to hepatocytes (bar 2), with control cells recieved vehicle (H_2O) (bar 1).

KCM: Hepatocytes were treated with medium taken from Kupffer cells, which had been treated with vehicle (bar 3) or with LPS (bar 4) (50 - 100 μg/ml) for 24 hours.

The mean and standard deviation for controls was: 4.37 ± 2.47 pmol/mg/min.

n = 20 using 5 homogeneous pools, except bar 2 where n = 8 from 2 pools, each obtained from 4 to 8 mice.

* significant differences from control (adjacent open bar). $p < 0.05$.

16) hours of exposure to the hepatocytes. Medium from Kupffer cells treated with water had no effect on CYP1A activity.

To reduce the chance of loosened Kupffer cells, or infections from being transferred from the Kupffer cell medium to the hepatocytes, the experiment was repeated, with the conditioned medium passed through a 0.2 μm filter before being added to the hepatocytes. This did not prevent the medium from Kupffer cells exposed to LPS (100 μg/ml) from decreasing the CYP1A activity compared to control cells,or cells treated with medium from Kupffer cells treated with water at 10 hours (fig. 14) or 24 hours (fig. 15). LPS (100 μg/ml), when added directly to hepatocyte plates, produced no response at 10 hours (fig. 12, and 14), or at 24 hours (fig. 13, fig. 15, and fig. 16).

3.3.2 *In vivo* Treatment with Deoxynivalenol

The effect of the mycotoxin deoxynivalenol, or vomitoxin, on CYP1A was investigated because it is reported to increase the mRNA coding for a variety of cytokines, including those implicated in the alterations of P-450. This change in the level of mRNA coding for these cytokines was reported to correspond to changes in cytokine levels, although those levels were not determined. In the present experiments, vomitoxin had no effect on P-4501A when administered orally to mice (Fig 17 and 18).

3.3.3 Kupffer Cell Conditioned Medium Produced by Deoxynivalenol

No effect on CYP1A was observed when the mycotoxin was incubated directly with hepatocytes (Fig. 19), or when medium from Kupffer cells which had been exposed to the mycotoxin, was applied to the hepatocytes (Fig. 19). LPS treated Kupffer cell

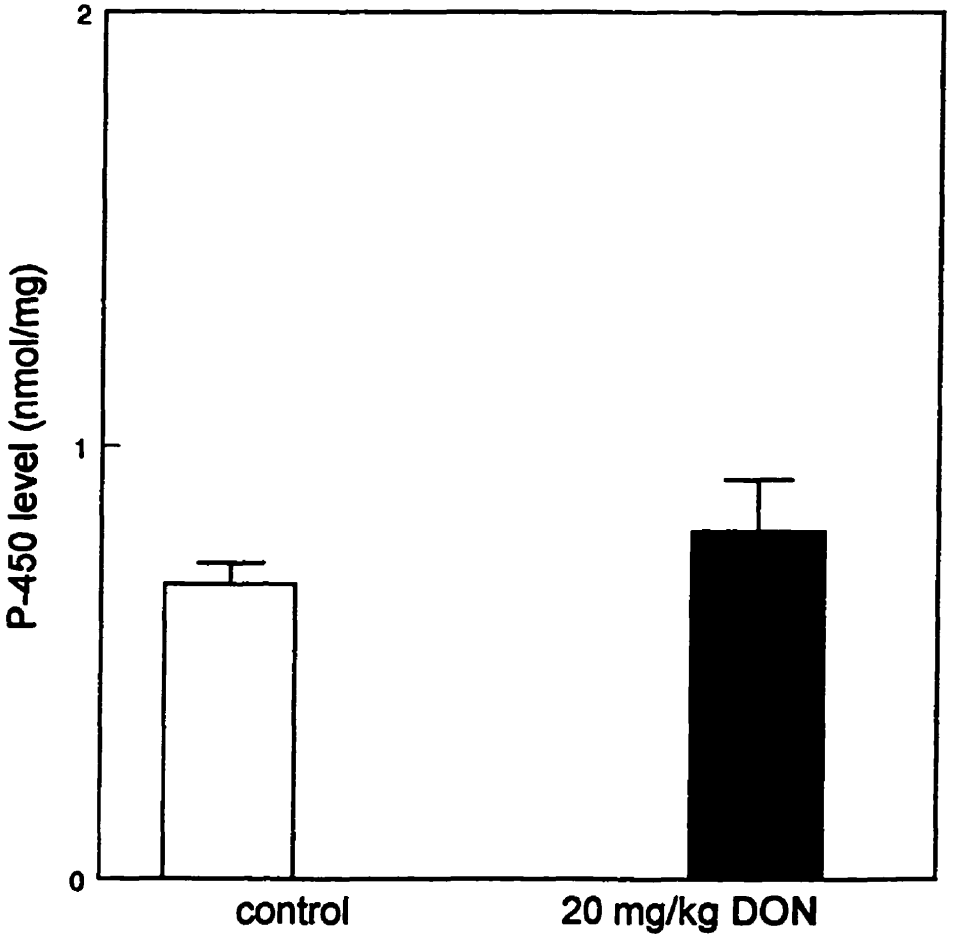


FIGURE 17

Effect of Deoxynivalenol (DON) on total microsomal cytochrome P-450 level.
Cytochrome P-450 level (nmol/mg protein), as determined by carbon monoxide binding spectrum, was found in microsomes prepared from mice which had been administered vehicle, or 20 mg/kg DON orally.
n = 6 for each group.
Student t-test. $p < 0.05$.

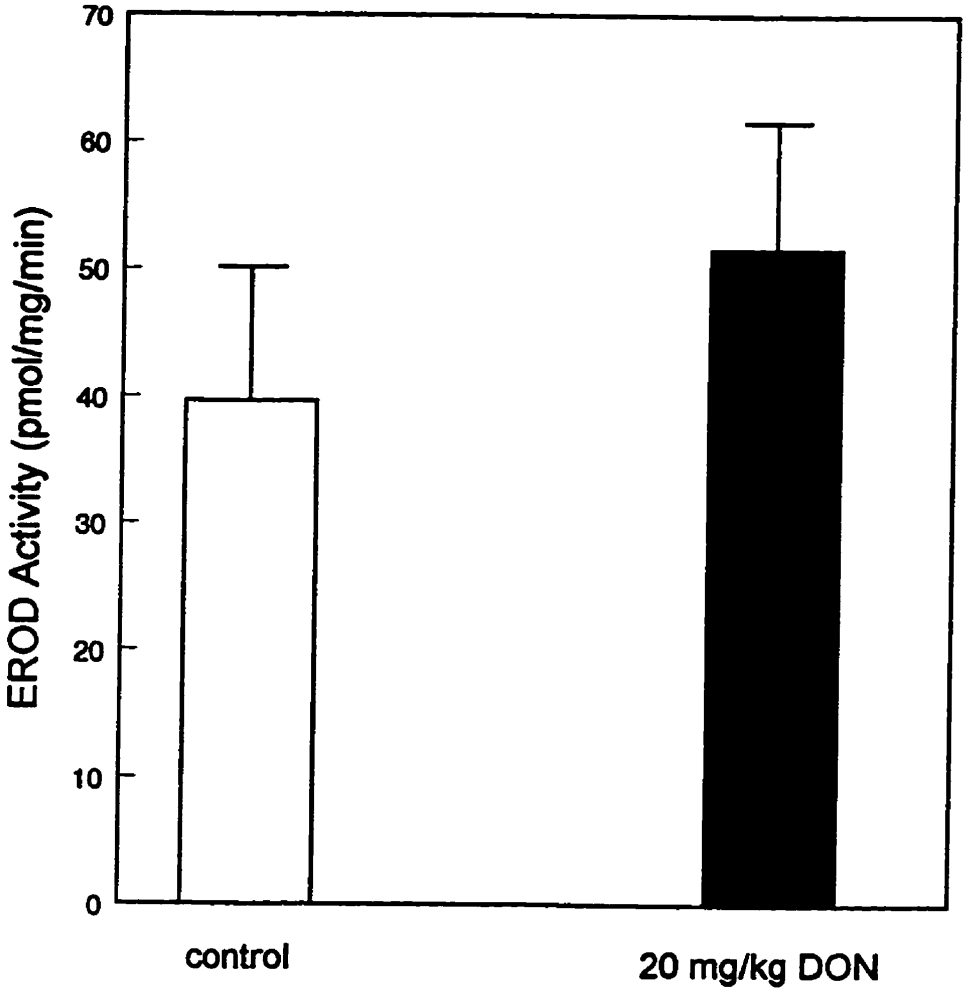


FIGURE 18

Effect of Deoxynivalenol (DON) on microsomal CYP1A activity.
CYP1A (pmol/mg/min), as determined by ethoxyresorufin-o-demethylase activity, was found in microsomes prepared from mice which had been administered vehicle, or 20 mg/kg DON orally.
n = 6 for each group.
Student t-test. $p < 0.05$.

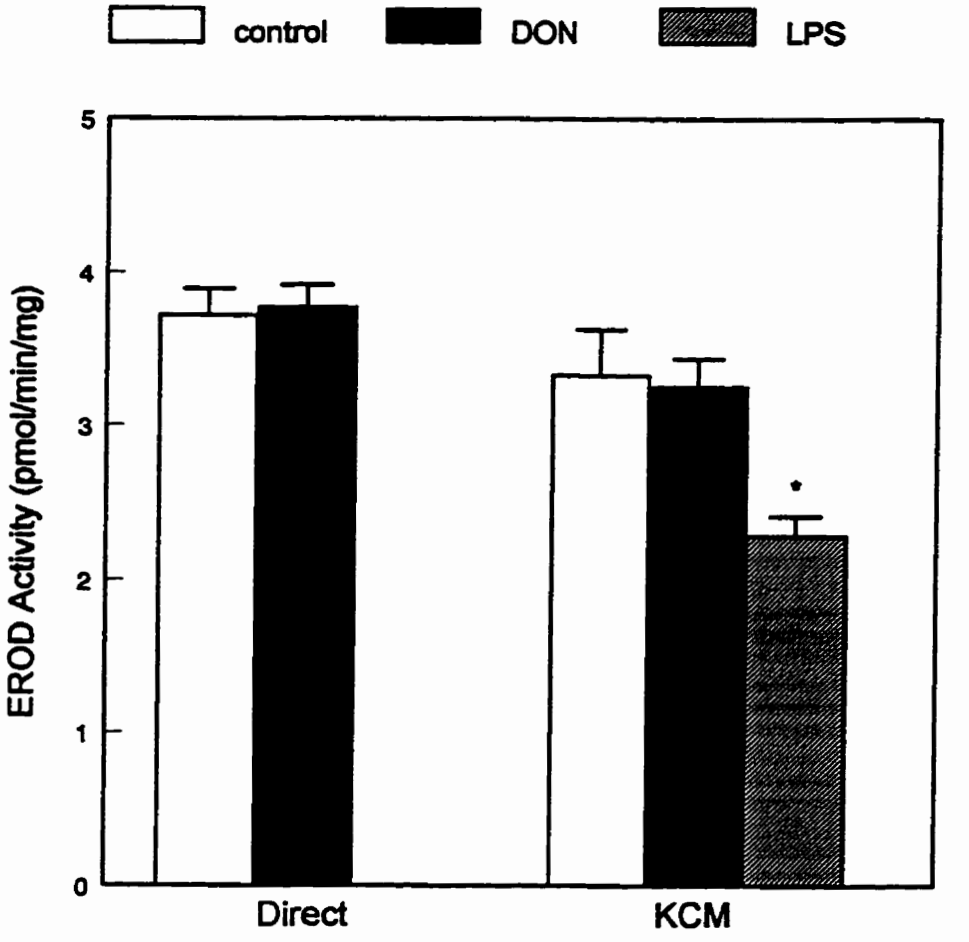


FIGURE 19

Effect of Deoxynivalenol (DON) on CYP1A activity in isolated hepatocytes.

CYP1A was induced in culture by the addition of DBA to the culture medium.

DIRECT: Cells were treated with water (bar 1), or 75 µg/ml DON (bar 2).

KCM: Cells were treated with 20 % fresh medium/80% conditioned medium. The conditioned medium was obtained from Kupffer cells which had been treated for 24 hours with water (bar 3), 75 µg/ml DON (bar 4), or 50 µg/ml LPS (bar 5).

n = 4, using 1 homogenous pool, obtained from 4 to 8 mice.

* significant difference from control (adjacent open bar).

conditioned medium was included in this experiment, to serve as a positive control for the responsiveness of the Kupffer cells. This medium from Kupffer cells treated with LPS resulted in a decrease in P-4501A relative to control.

3.3.4 Direct TNF-α Stimulation

Many cytokines are known to cause a decrease in the levels of P-450. LPS treated Kupffer cell conditioned medium is reported to contain a variety of cellular mediators, including cytokines, but the exact contents, and concentrations within the medium obtained in these studies was not measured, and was likely highly variable between experiments due to variations in cell densities. In order to investigate the effects of a single known cytokine, TNF (10 ng/mL) was added to DBA induced hepatocytes. It was also an objective within this study to look at the time course of TNF mediated decrease in CYP1A activity. In a representative study, TNF stimulation resulted in a reduction of CYP1A activity within 6 hours of stimulation, and this remained depressed following 12 hours and 24 hours of TNF stimulation (Fig. 20). Compiling the data from several experiments (6 for 4-6 hours, 16 for 24 hours), it was shown TNF did not result in a decrease in CYP1A activity before the 6 hour time point (fig. 21), and produced a decrease in CYP1A activity at 24 hours of approximately 20% (fig. 21). This decrease in activity did not appear to correspond to a decrease in CYP1A1 protein as determined by Western immunoblot analysis (fig. 22).

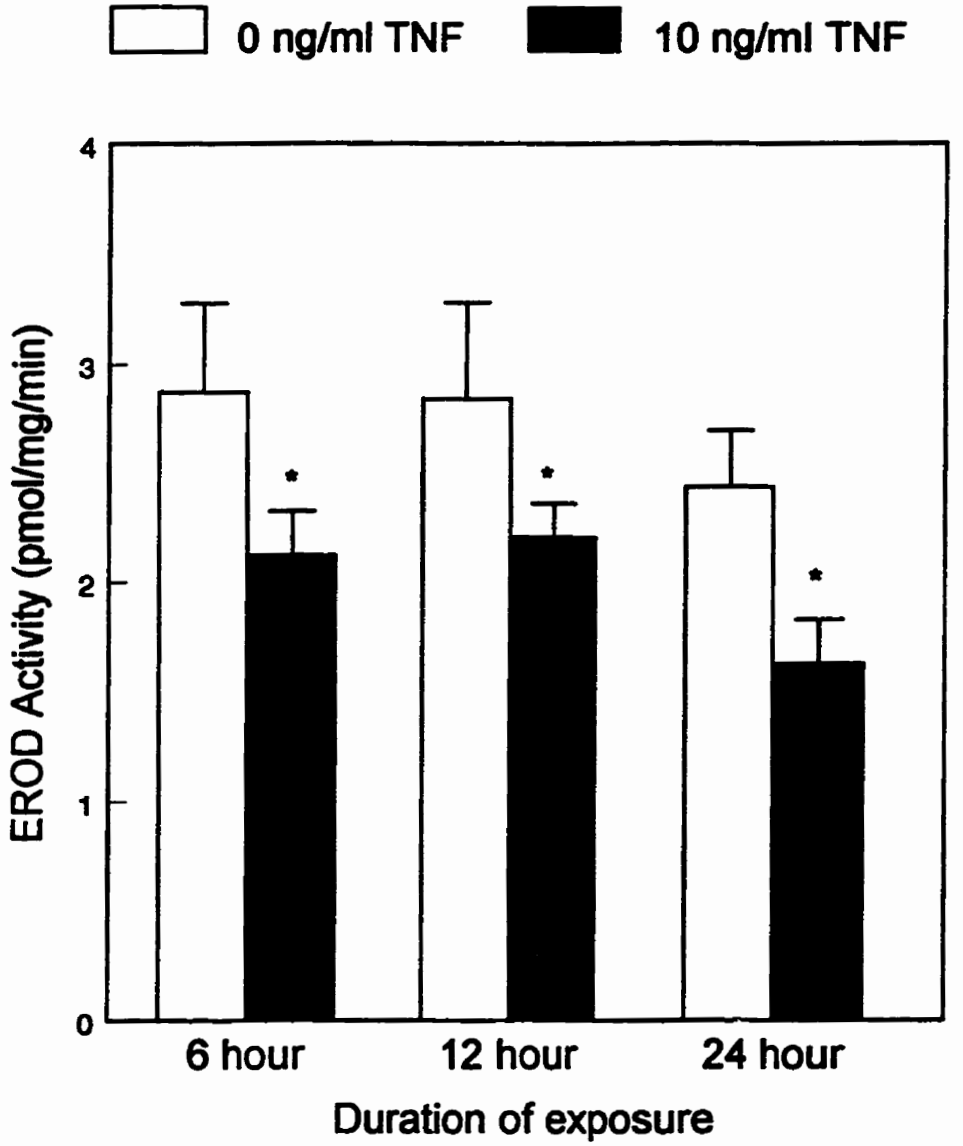


FIGURE 20

Effect of TNF on CYP1A activity in isolated hepatocytes.
CYP1A was induced in hepatocytes by the addition of DBA (50 nM) to the plated cells for 24 hours. TNF (10 ng/ml) was added for the duration indicated.
n = 4, using 1 homogenous pool obtained from 4 to 8 mice.
*significant difference from control (adjacent open bar). $p < 0.05$.

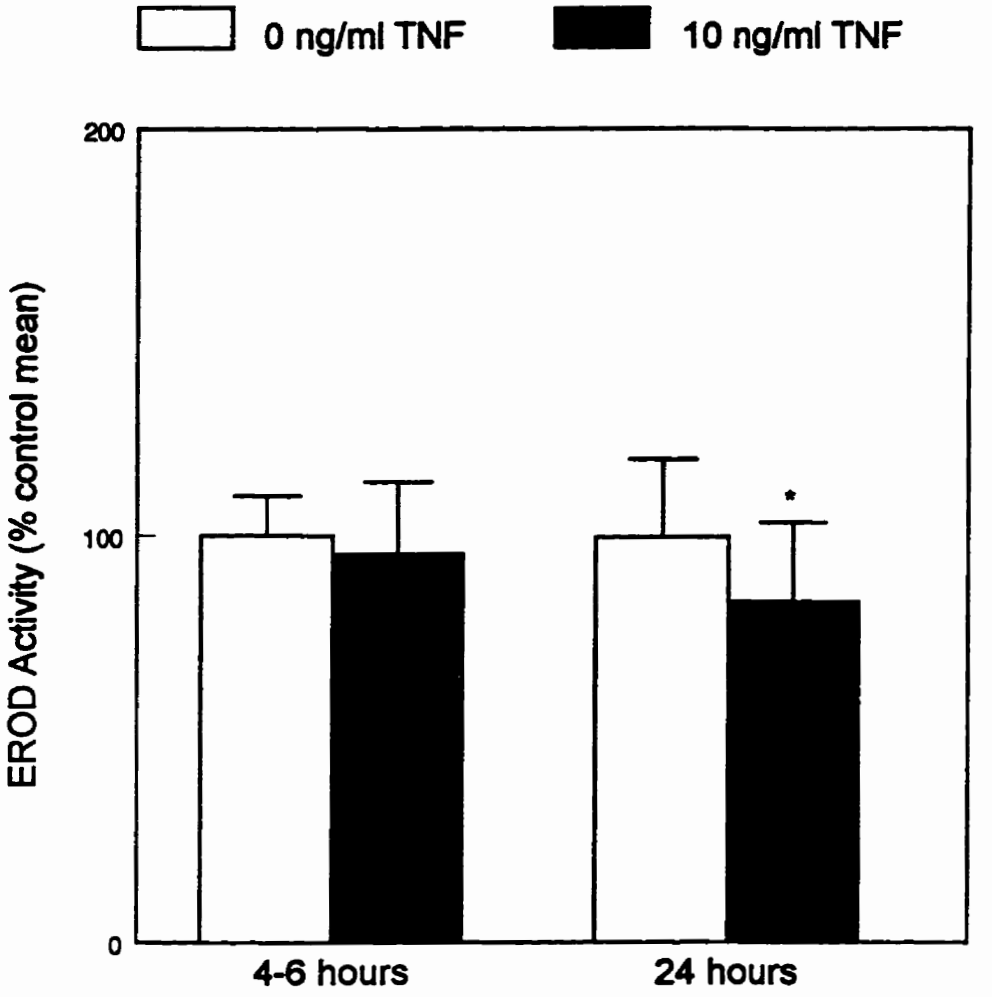
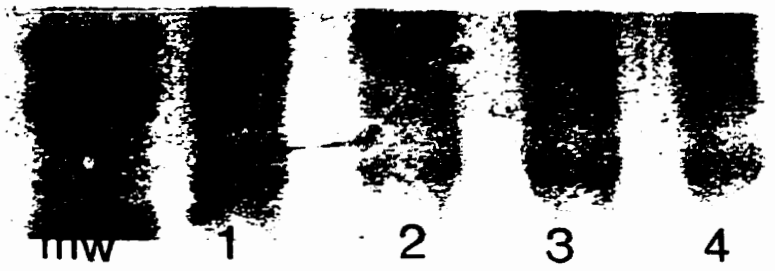


FIGURE 21

Effect of TNF on CYP1A activity in isolated hepatocytes (pooled).
CYP1A activity was induced by the addition of DBA (50 nM) to plated cells for 24 hours. TNF (10 ng/ml) was added for the duration indicated.
* significant difference from control (adjacent open bar).$p < 0.05$.
The average and standard deviation for control (24 hr) was: 6.13 ± 3.27 pmol/mg/min.
4-6 hour group: n = 24, using 6 homogenous pools,
each obtained from 4 to 8 mice.
24 hour group: n = 64, using 16 homogenous pools,
each obtained from 4 to 8 mice.



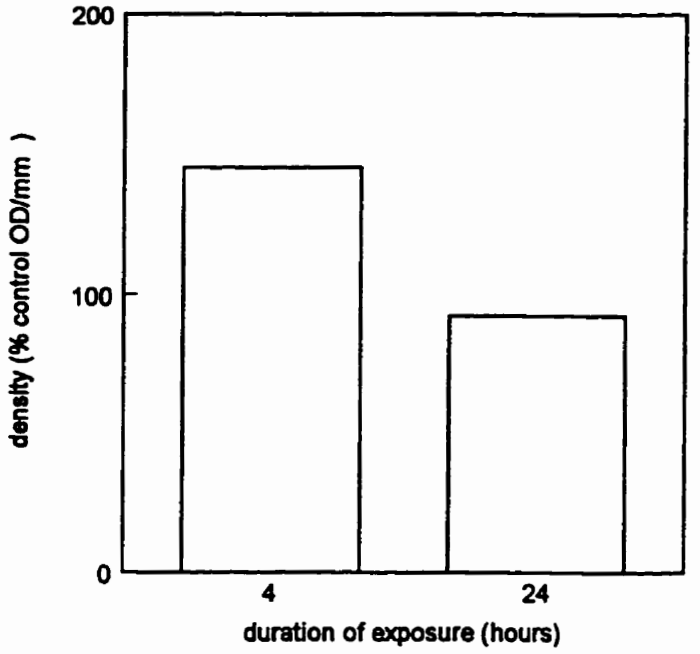


FIGURE 22

Effect of TNF on CYP1A protein
Western immunoblot analysis of CYP1A1 levels following 4 hour and
24 hour treatment with TNF. CYP1A was induced with DBA.
Lanes: mw = molecular weight marker
1= control 4 hour, 2 = TNF 4 hour,
3= control 24 hour, 4 = TNF 24 hour.
Bars represent percent of the control density, as determined by densitometry. 4 hour time point is from one pool, 24 hour time point an average of 2 pools.

3.4 THE ROLE OF PROTEIN SYNTHESIS IN CYTOKINE MEDIATED DECREASES IN CYP1A

3.4.1 Protein Synthesis Inhibitors *in vitro*

A requirement for protein synthesis to occur to achieve the decrease in cytochrome P-450 has been suggested from experiments conducted in whole animals. To study this aspect, protein synthesis inhibitors actinomycin D, an inhibitor of transcription and puromycin, an inhibitor of translation were chosen, as they have been shown to be effective at blocking the effect of IFN on cytochrome P-450 in vivo. In cells with CYP1A induced with DBA, both Actinomycin D and Puromycin, caused a significant depression in CYP1A activity after 24 hours (fig. 23, fig. 25). There was no further decrease in the activity when TNF was added in the presence of either inhibitor (fig. 23, fig. 25). In the case of cells obtained from mice which had been pre-induced with BNF in order to elevate CYP1A levels, the addition of the protein synthesis inhibitors, Actinomycin D and Puromycin, had no effect. TNF in the presence of these inhibitors did not depress CYP1A activity relative to the protein synthesis inhibitor alone, or relative to control cells (fig 24, fig. 26).

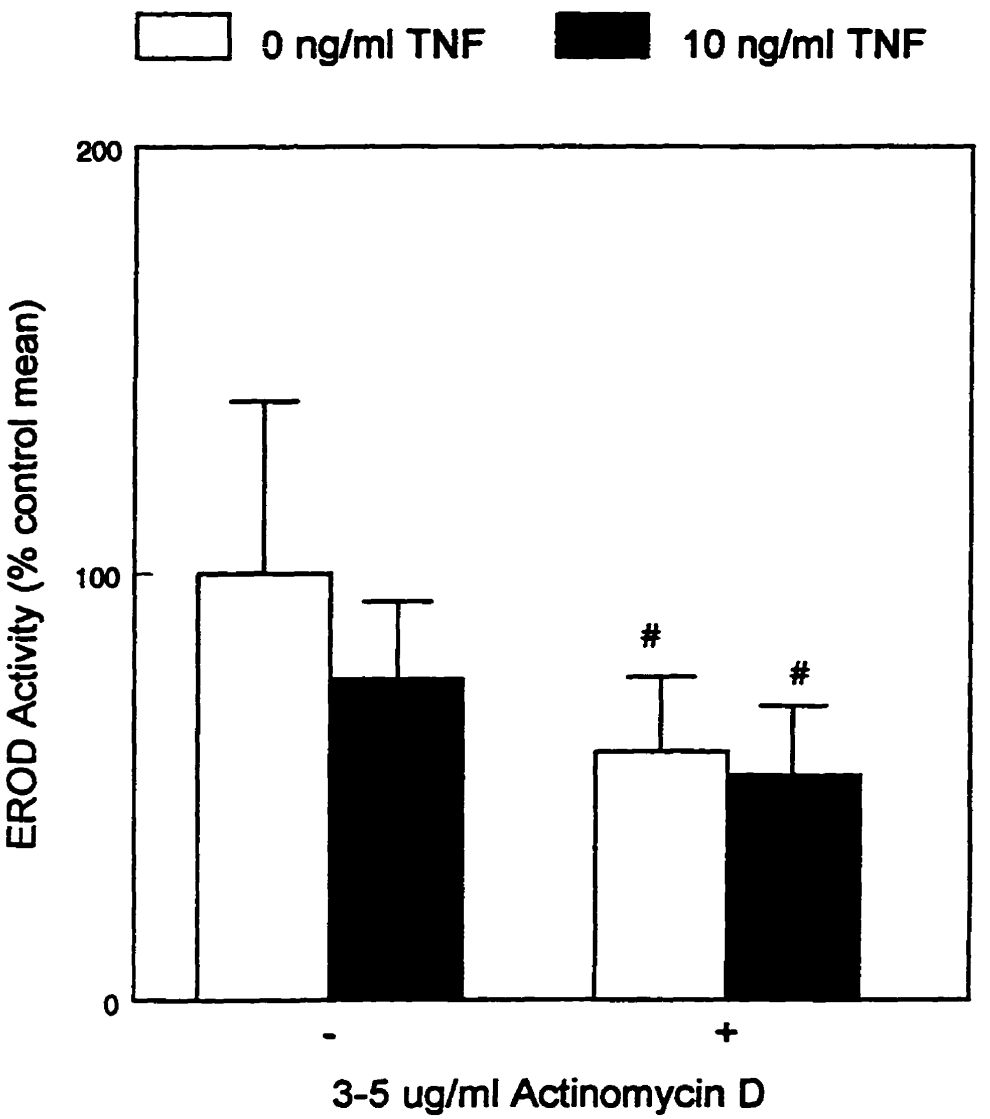


FIGURE 23

Effect of the protein synthesis inhibitor Actinomycin D (3-5 ug/ml) on TNF mediated depression of DBA induced CYP1A activity.
Mean and standard deviation for controls was: 5.83 ● 2.99 pmol/mg/min.
n = 8, using 2 homogenous pools, each obtained from 4 to 8 mice.
* significant difference from control (adjacent open bar). $p < 0.05$.
# significant difference from untreated control (bar 1). $p < 0.05$.

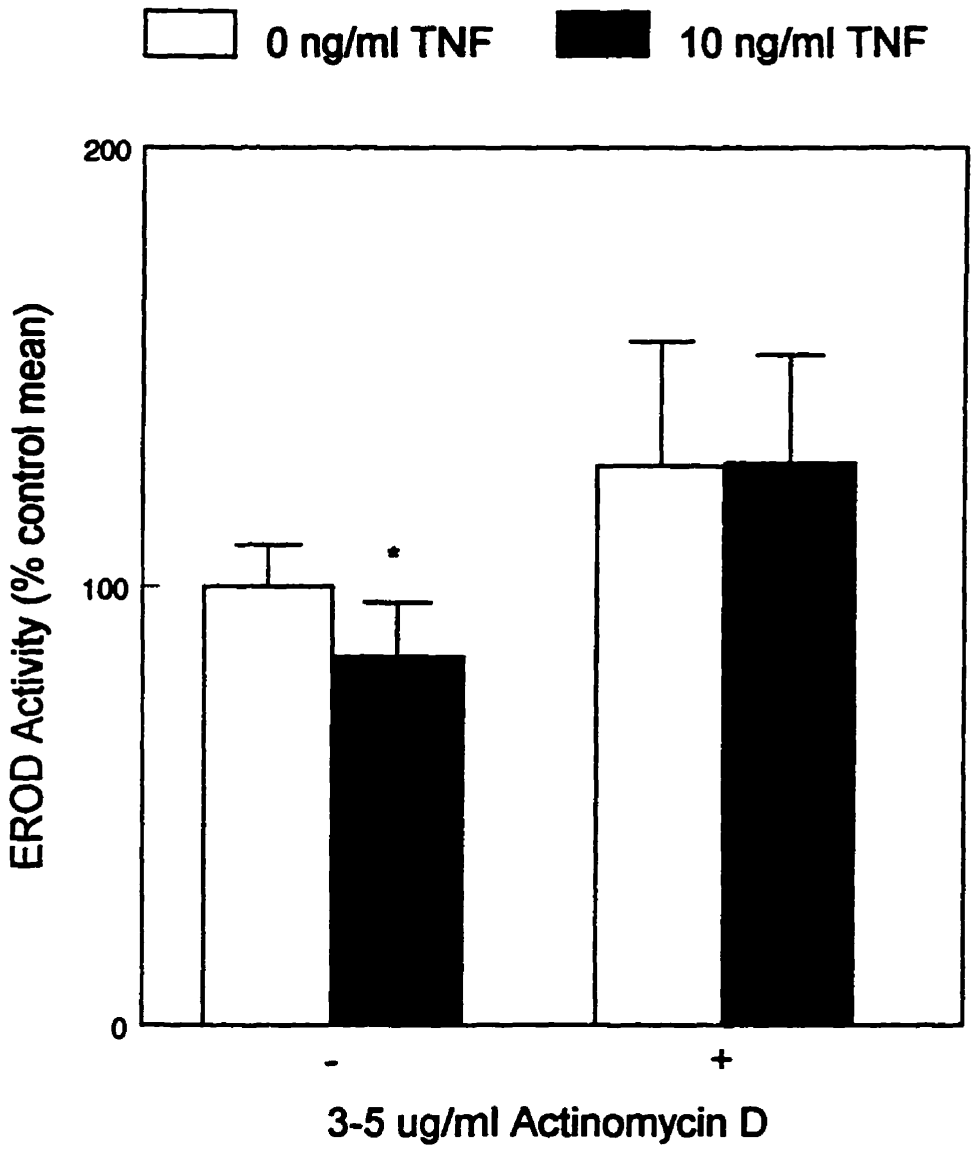


FIGURE 24

Effect of the protein synthesis inhibitor Actinomycin D (3-5 ug/ml) on TNF mediated depression of BNF induced CYP1A activity.
Mean and standard deviation for controls was: 3.63 ± 0.50 pmol/mg/min.
n = 16, using 4 homogenous pools, each obtained from 4 to 8 mice.
* significant difference from control (adjacent open bar). $p < 0.05$.

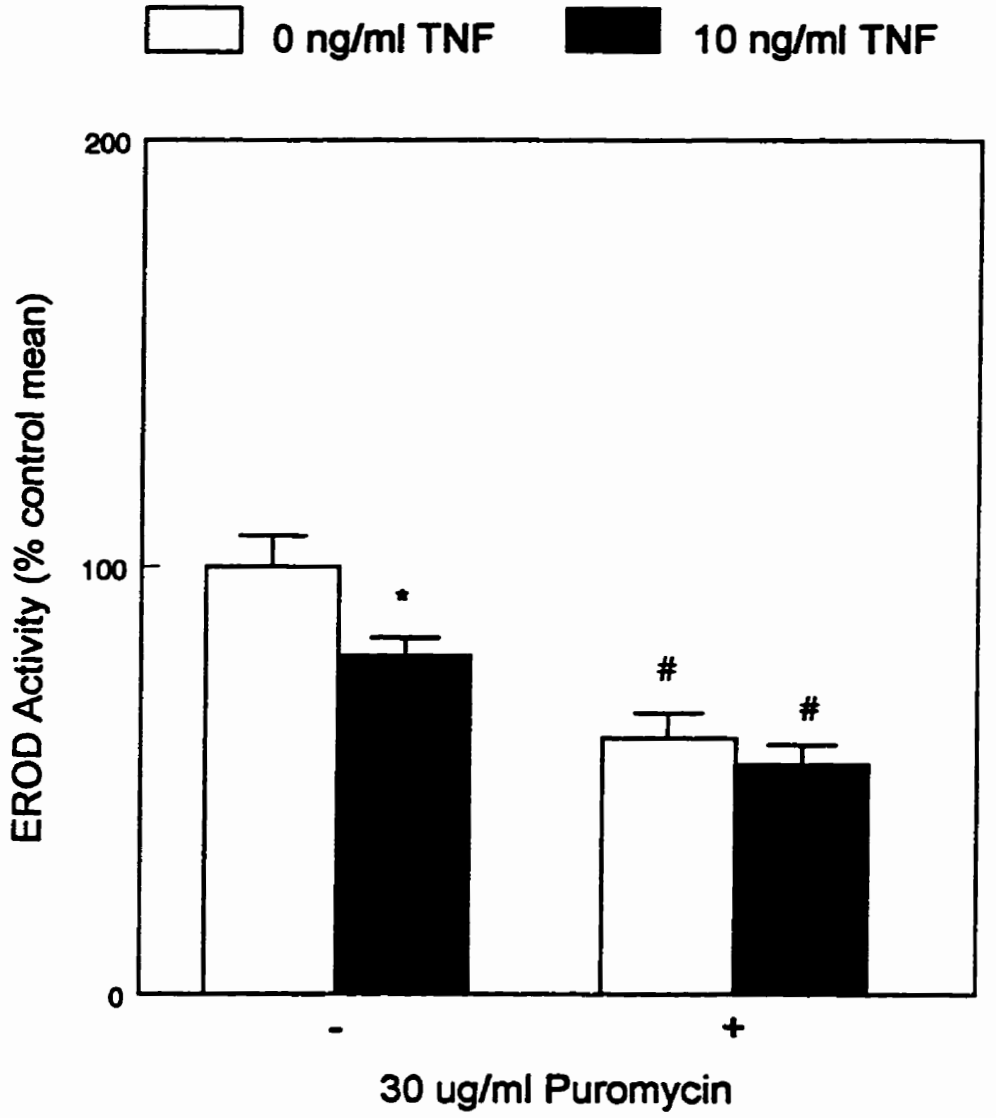


FIGURE 25

Effect of the protein synthesis inhibitor Puromycin (30 ug/ml) on TNF mediated depression of DBA induced CYP1A activity.
Mean for controls was: 9.729 pmol/mg/min.
n = 4, using 1 pool, obtained from 4 to 8 mice.
* significant difference from control (adjacent open bar). $p < 0.05$
# significant difference from untreated controls (bar 1). $p < 0.05$.

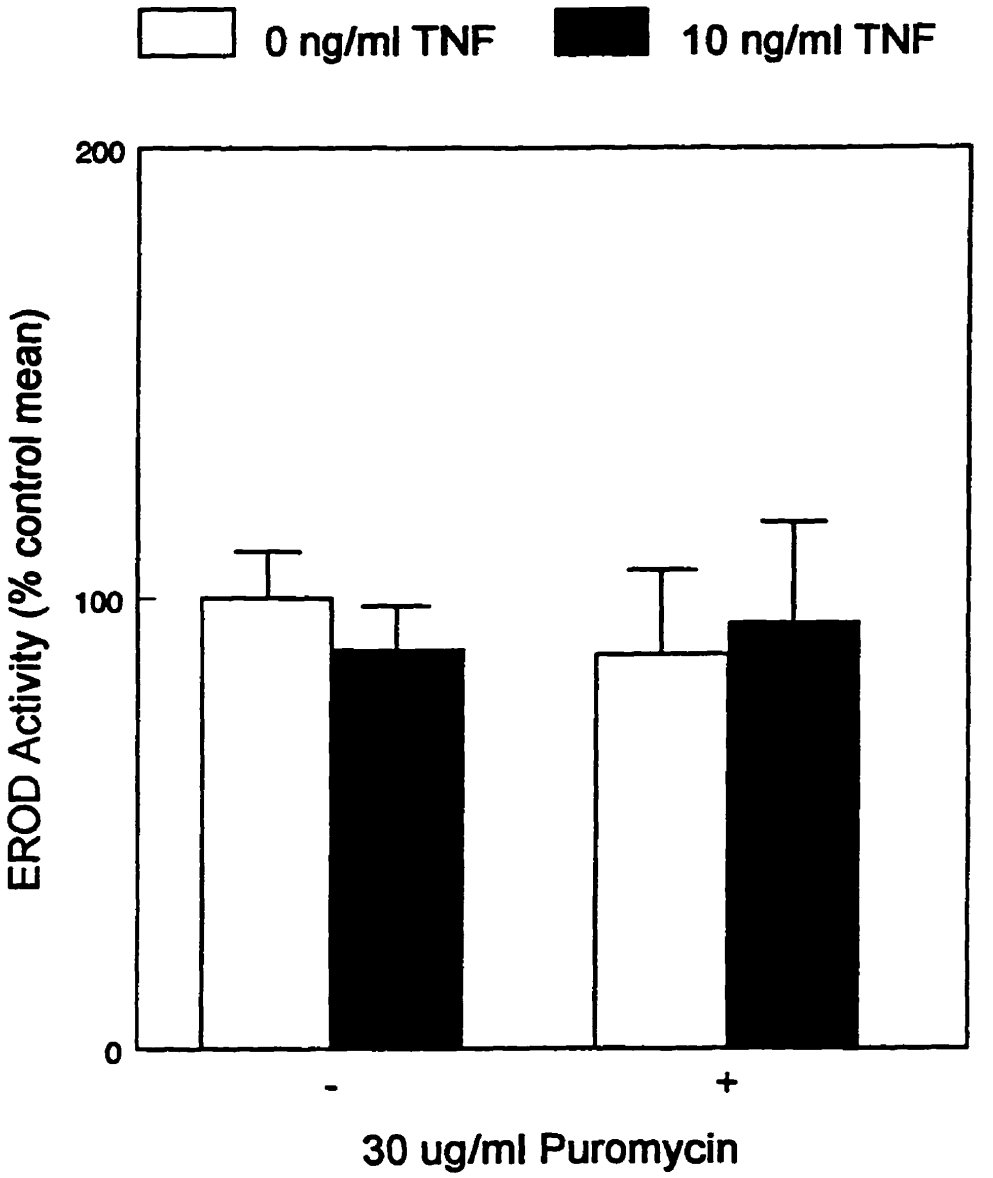


FIGURE 26

Effect of the protein synthesis inhibitor Puromycin (30 ug/ml) on TNF mediated depression of BNF induced CYP1A activity.
Mean and standard deviation for controls was: 3.52 ● 0.54 pmol/mg/min.
n = 12, using 3 homogenous pools, each obtained from 4 to 8 mice.

3.5 THE ROLE OF NITRIC OXIDE IN CYTOKINE MEDIATED DECREASES IN CYP1A

3.5.1 Nitric Oxide Generating Compound

There is substantial evidence to suggest that nitric oxide may be involved in the pathway leading to the decrease in cytochrome P-450 activity following activation of host defense, or administration of cytokines. Hepatocytes were exposed to the nitric oxide generator, sodium nitroprusside at a range of concentrations and times. Although 0.12 mM SNP was not sufficient to cause a decrease in CYP1A at any of the time points examined, concentrations of 1.2mM, and 12 mM caused a decrease within 30 minutes, which is the earliest time point that can be observed using this technique. Both 1.2 mM and 12 mM remained significantly different from control at 1 hour and 24 hour. A concentration of 12 mM, but not 1.2 mM was significantly different at the 4 hour time point (Fig. 27). A combination of two separate experiments, one which included the 4 and 24 hour time points, the other included the 0.5 and 1 hour time points, is shown in figure 28. Western immunblot analysis indicates that the decrease in CYP1A activity in response to SNP is not due to a decrease in CYP1A1 protein levels even at the 24 hour time point (fig. 29).

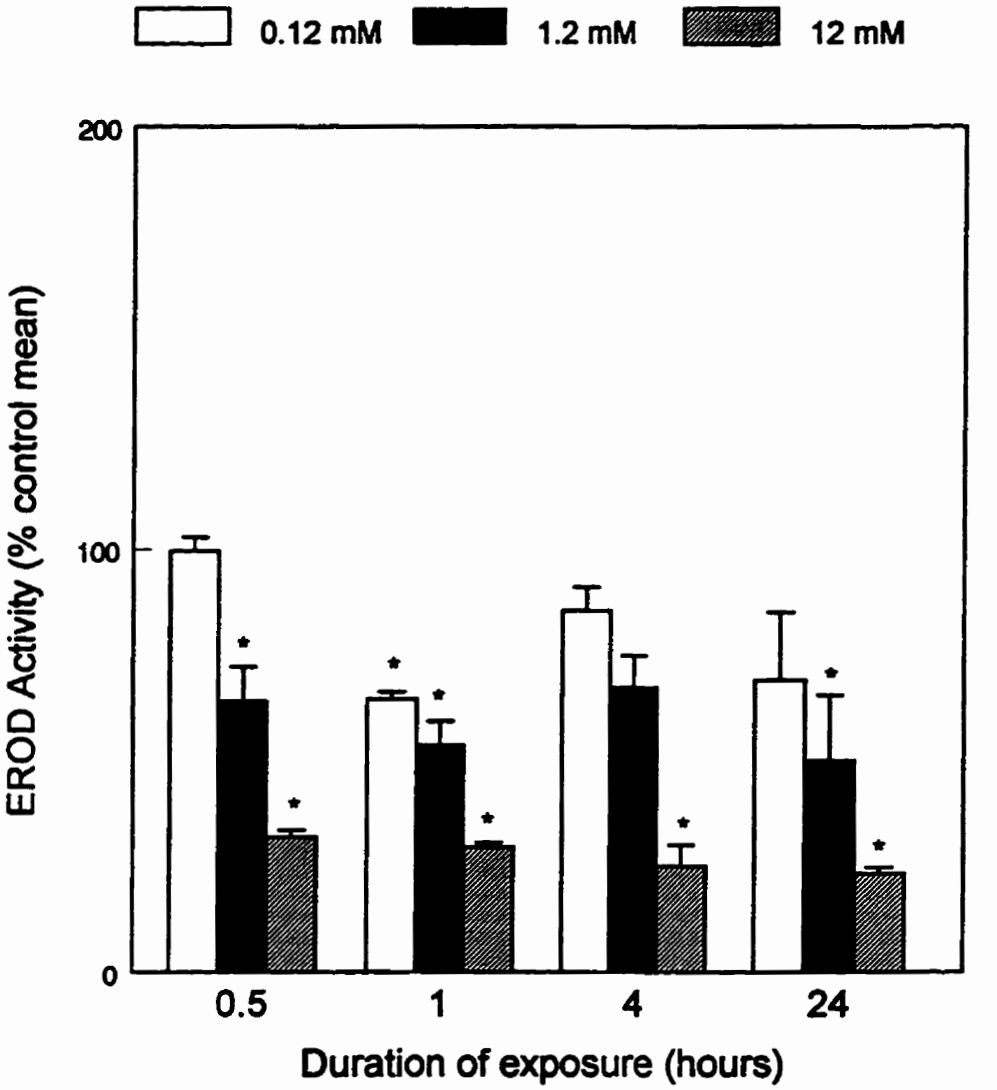


FIGURE 27

Effect of Sodium Nitro Prusside (SNP) on CYP1A activity
CYP1A activity was induced with DBA
Sodium nitroprusside was added in three concentrations;
0.12 mM open bars, 1.2 mM solid bars, and 12 mM hatched bars
for four lengths of time; 0.5 hours, 1 hour, 4 hours, and 24 hours.
n = 4 using 1 homogenous pool obtained from 4 to 8 mice.
* significant difference from control (bar not shown). $p < 0.05$.

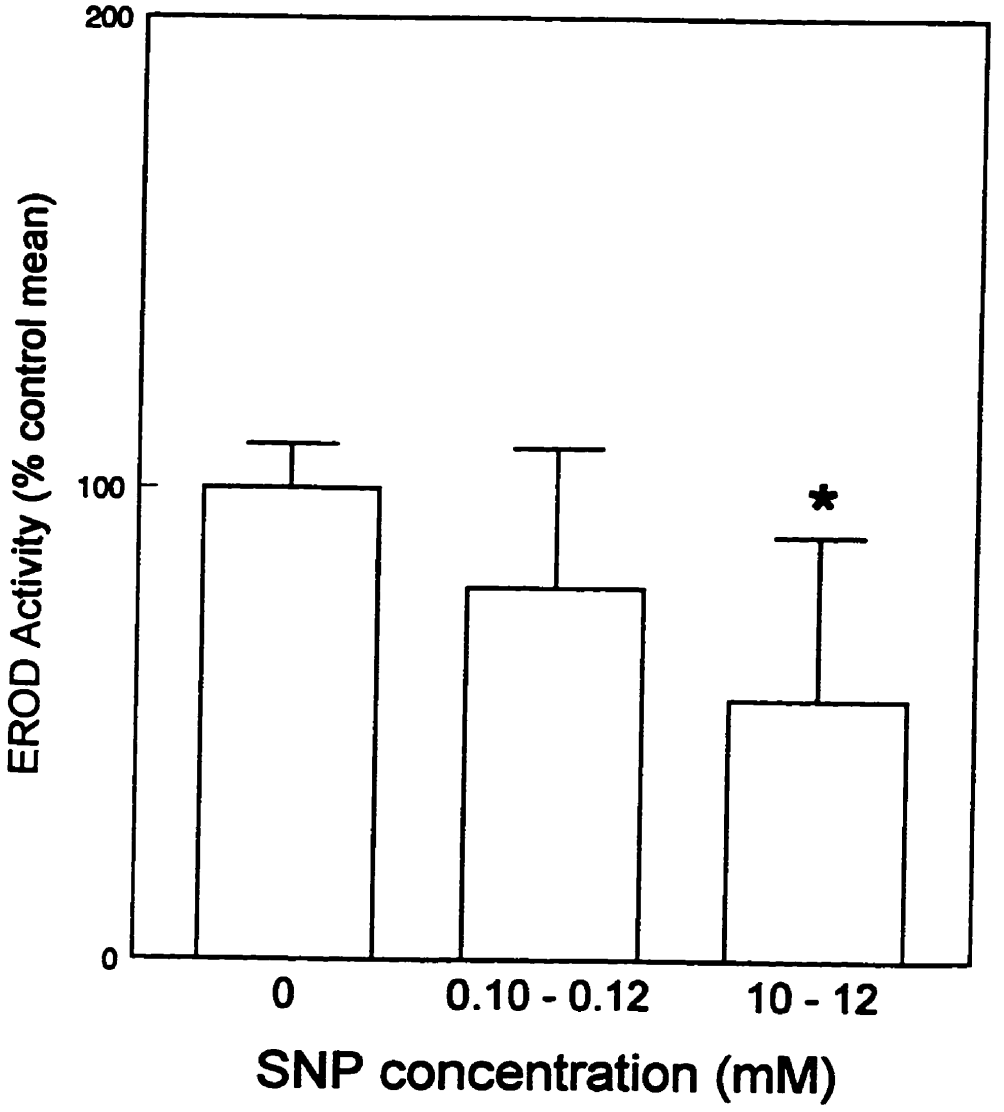


FIGURE 28

Effect of Sodium Nitroprusside on CYP1A activity (pooled).
Mean of control was: 6.90 ± 6.65 pmol/mg/min.
n = 12, using 3 homogenous pools, each obtained from 4 to 8 mice.
* significant difference from control (adjacent open bar). $p < 0.05$.

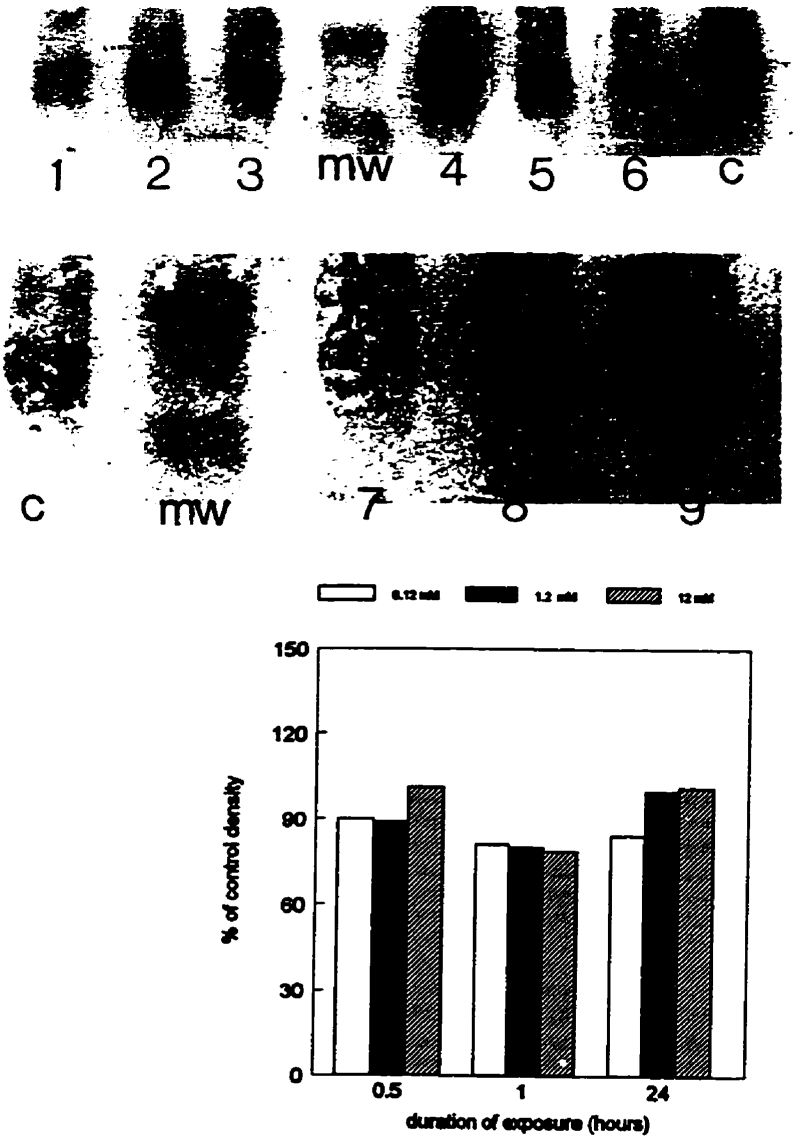


FIGURE 29

Effect of SNP on CYP1A protein
Western immunoblot analysis of one pool, repeated twice.
Lanes: mw = molecular weight marker
1, 2, and 3: 1 hour
4, 5, and 6: 0.5 hour
7, 8 and 9: 24 hour
1, 4 and 9 = 12 mM
2, 5 and 8 = 1.2 mM
3, 6 and 7 = 0.12 mM
Each bar represents the average percent of the control density, as determined by densitometry.

3.5.2 Inducible Nitric Oxide Synthase Inhibitor

While it was well established that nitric oxide donors are able to produce a decrease in activity, what is not so clear is the involvement of nitric oxide in cytokine mediated depression of cytochrome P-450 activity. The general nitric oxide synthase inhibitor, NNA, and two nitric oxide synthase inhibitors selective for the inducible form of the enzyme (NOS2), NIO and NIL, were used to investigate the involvement of nitric oxide synthase in the TNF mediated decrease in P-4501A activity (fig. 30, 31, 32, and 33). NIL at 100 μM was effective at blocking the depression of CYP1A by TNF (fig. 32). In all cases, the presence of a nitric oxide synthase inhibitor eliminated any significant difference reduction in CYP1A activity following the addition of TNF.

3.6 THE ROLE OF NF-κB IN CYTOKINE MEDIATED DECREASES IN CYP1A

3.6.1 NF-κB Inhibition *in vitro*

To determine if NF-κB activation is required for the effect of TNF on CYP1A, the NF-κB inhibitors BTEE and TPCK were used to block the activation of NF-κB. In the presence of 1-2 mM BTEE, the addition of 10 ng/ml TNF did not produce a significant difference (fig. 34). This inhibitor appears to be effective at blocking the action of TNF on CYP1A, however caution concerning the relevance of this finding should be taken, as there was an increase in the standard deviation in the presence of the drug. Although there is no significant difference between TNF treated cells and their control in the

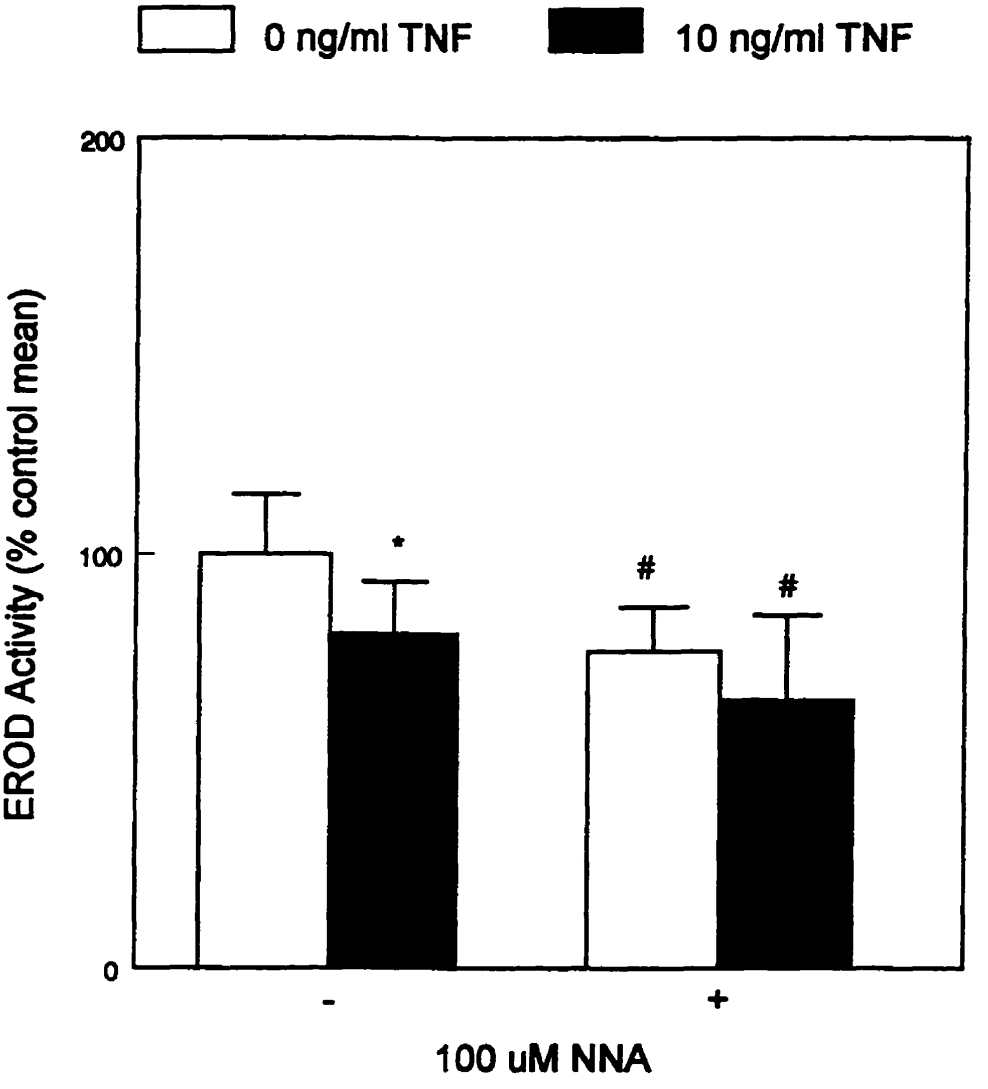


FIGURE 30

Effect of the nitric oxide synthase inhibitor, N-Nitro-Arginine (NNA) (100 uM) on TNF mediated depression CYP1A activity.
CYP1A was induced with DBA
Mean of control was: 5.16 ± 2.94 pmol/mg/min.
n = 8, using 2 homogenous pools, each obtained from 4 to 8 mice.
* significant difference from control (adjacent open bar). $p < 0.05$.
# significant difference from untreated control (bar 1). $p < 0.05$.

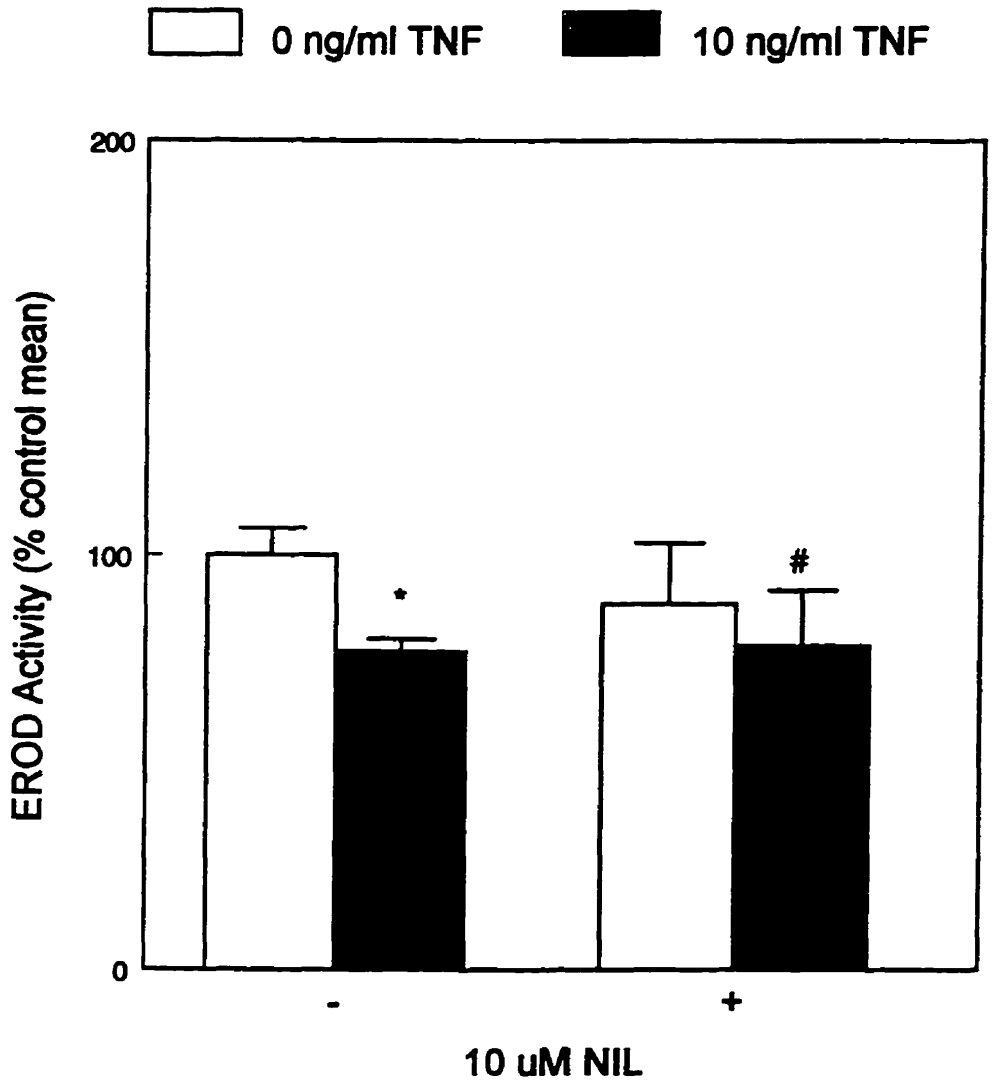


FIGURE 31

Effect of the nitric oxide synthase inhibitor, L-N6-(1-iminoethyl)lysine (NIL) (10 uM) on TNF mediated depression CYP1A activity.
CYP1A was induced with DBA.
Mean of control was: 7.39 pmol/mg/min.
n = 4, using 1 homogenous pool obtained from 4 to 8 mice.
* significant difference from control (adjacent open bar). $p < 0.05$.
# significant difference from untreated control (bar 1). $p < 0.05$.

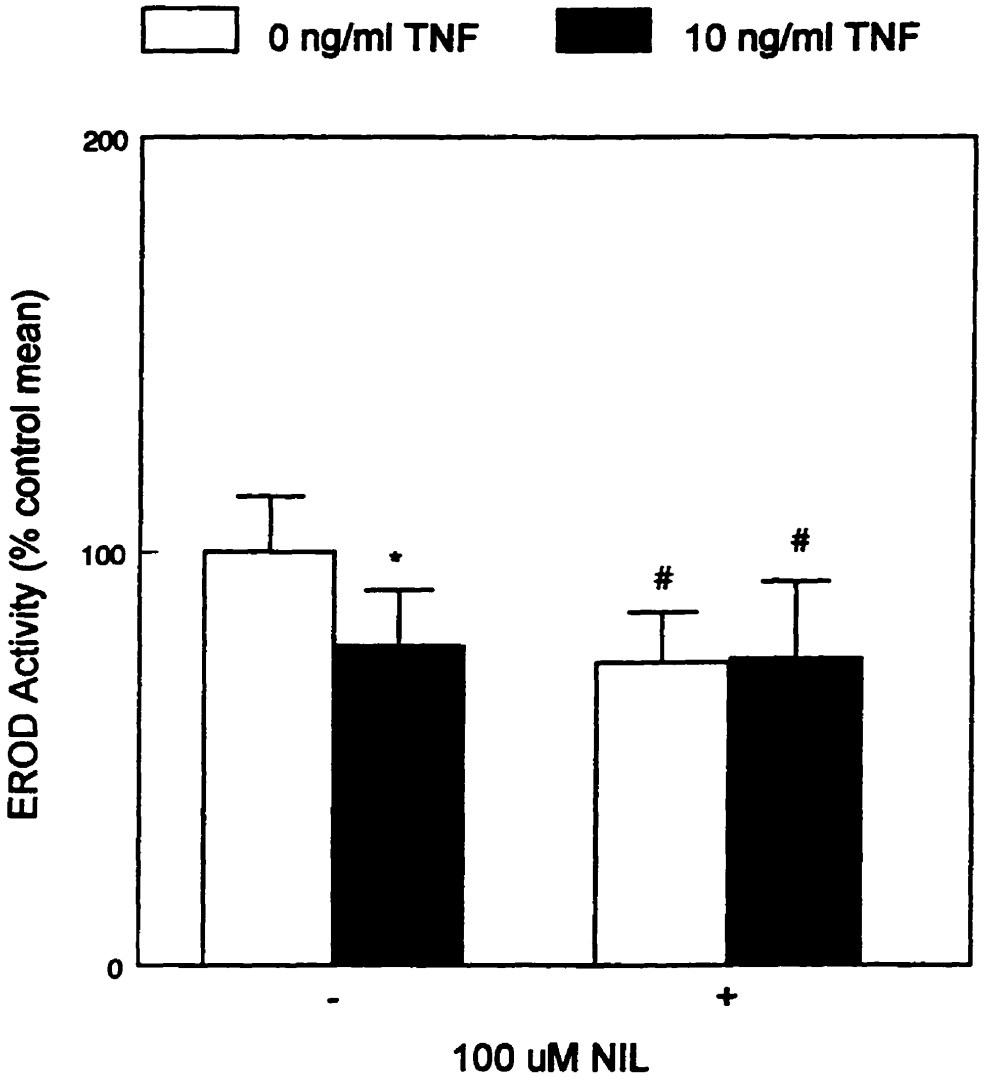


FIGURE 32

Effect of the nitric oxide synthase inhibitor, L-N6-(1-iminoethyl)lysine (NIL) (100 uM) onTNF mediated depression CYP1A activity.
CYP1A was induced with DBA
Mean of control was: 7.32 ± 0.11 pmol/mg/min.
n = 8, using 2 homogenous pools, each obtained from 4 to 8 mice.
* significant difference from control (adjacent open bar). $p < 0.05$.
# significant difference from untreated control (bar 1). $p < 0.05$.

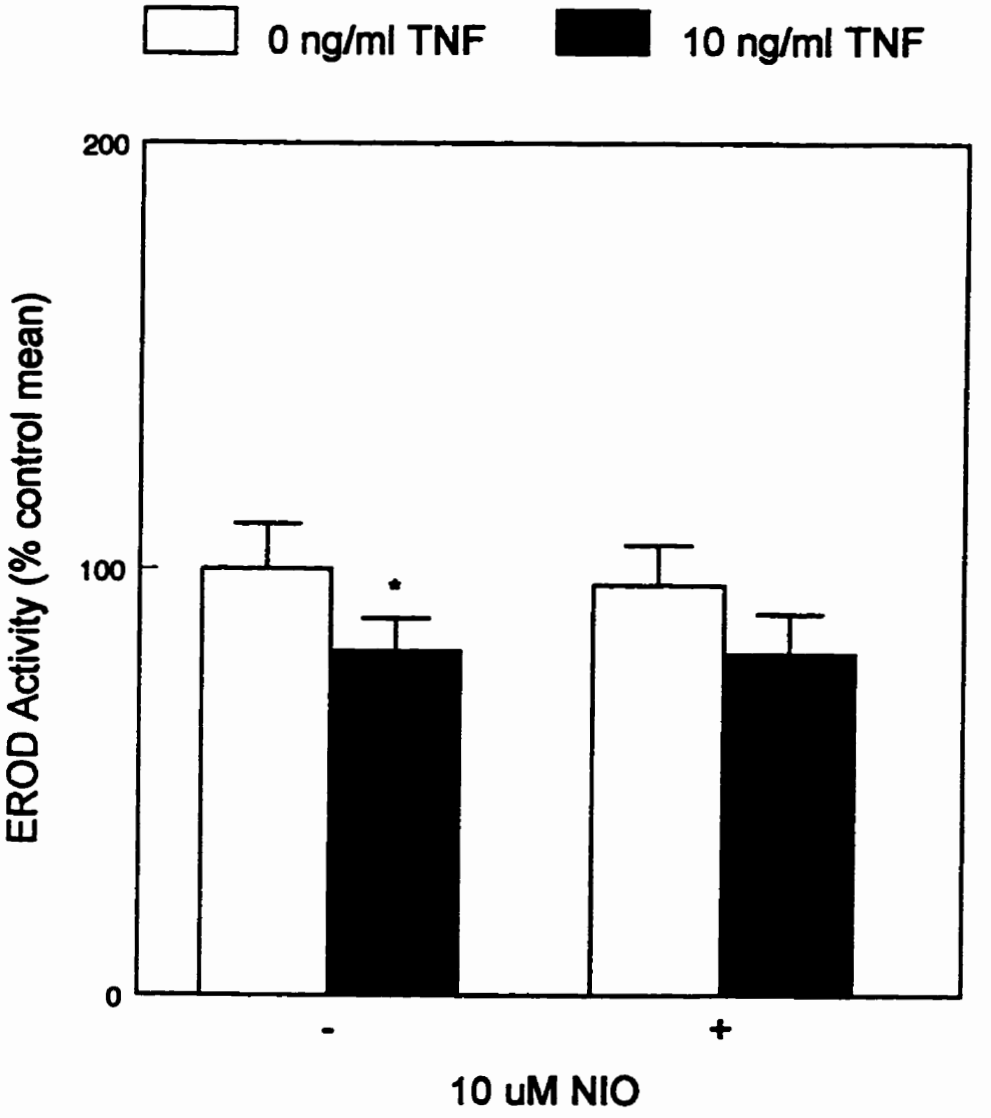


FIGURE 33

Effect of the nitric oxide synthase inhibitor, L-imino ornithine (NIO) (10uM) on TNF mediated depression of CYP1A activity.
CYP1A activity was induced with DBA.
Mean of control was: 4.88 pmol/mg/min.
n = 4, using 1 homogenous pool obtained from 4 to 8 mice.
* significant difference from control (adjacent open bar). $p < 0.05$.

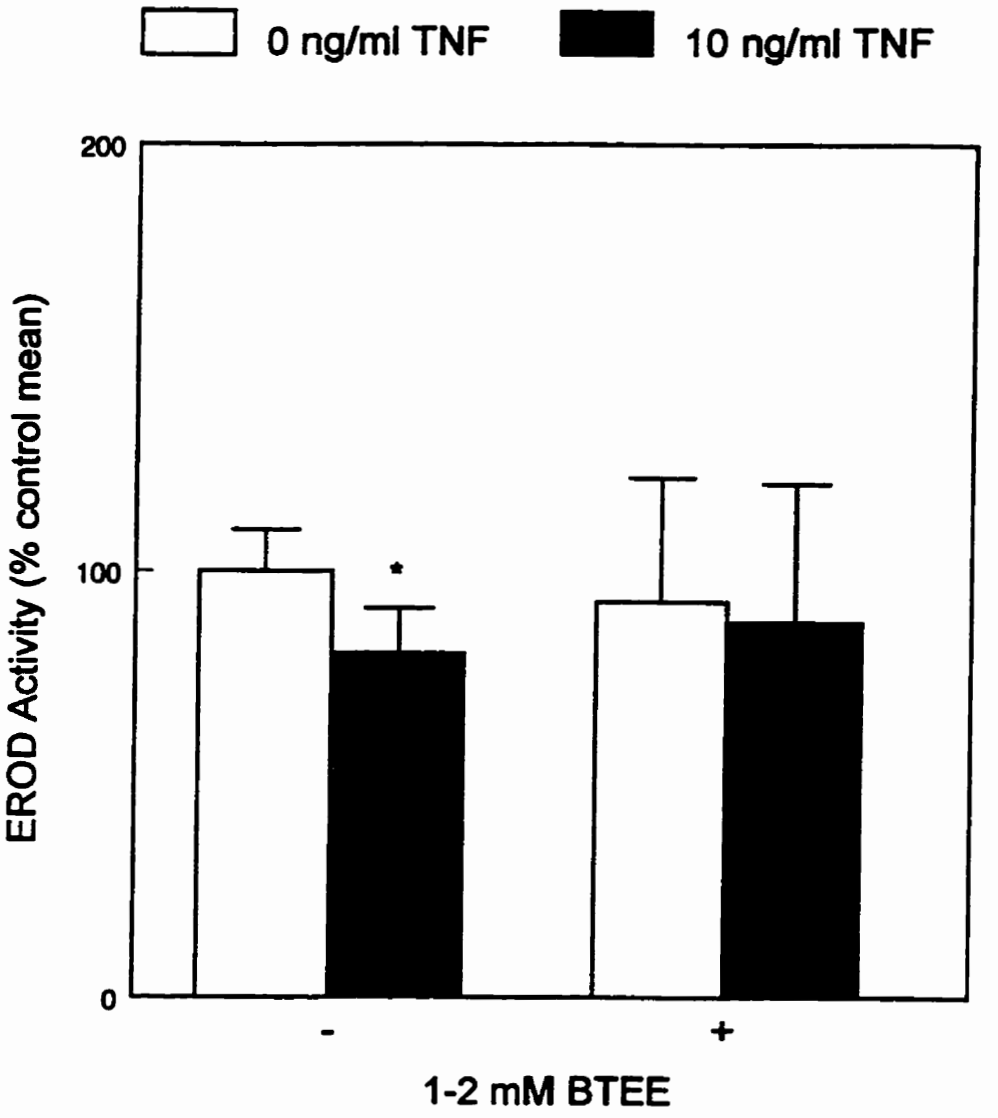


FIGURE 34

Effect of the NF-κB inhibitor, N-benzoyl-l-tyrosine ethyl ester (BTEE) (1-2 mM) on the TNF mediated depression of CYP1A activity.
CYP1A activity was induced with DBA.
Mean of control was:4.36 ± 2.55 pmol/mg/min.
n = 16, using 4 homogenous pools, each obtained from 4 to 8 mice
* significant difference from control (adjacent open bar). $p < 0.05$.

presence of the inhibitor TPCK at 10 μM or 100 μM (fig. 35 and fig. 36), indicating that the inhibitor was effective at blocking the TNF mediated depression of CYP1A activity, the difference was small to begin with, and the action of the drug appears to have more to do with the increase in the standard deviation in the presence of the drug, rather than a change in the effect of TNF. When the concentration of the NF-κB inhibitor TPCK was increased to 300 μM, it was an effective inducer itself of CYP1A, even in the absence of any other cytochrome P-450 inducer (fig. 37).

3.6.2 NF-κB Inhibition *in vivo*

IP administration of 30 mg/kg TPCK in ethanol to mice did not significantly increase total cytochrome P-450 content (fig. 38) or CYP1A activity (fig. 39) after 24 hours.

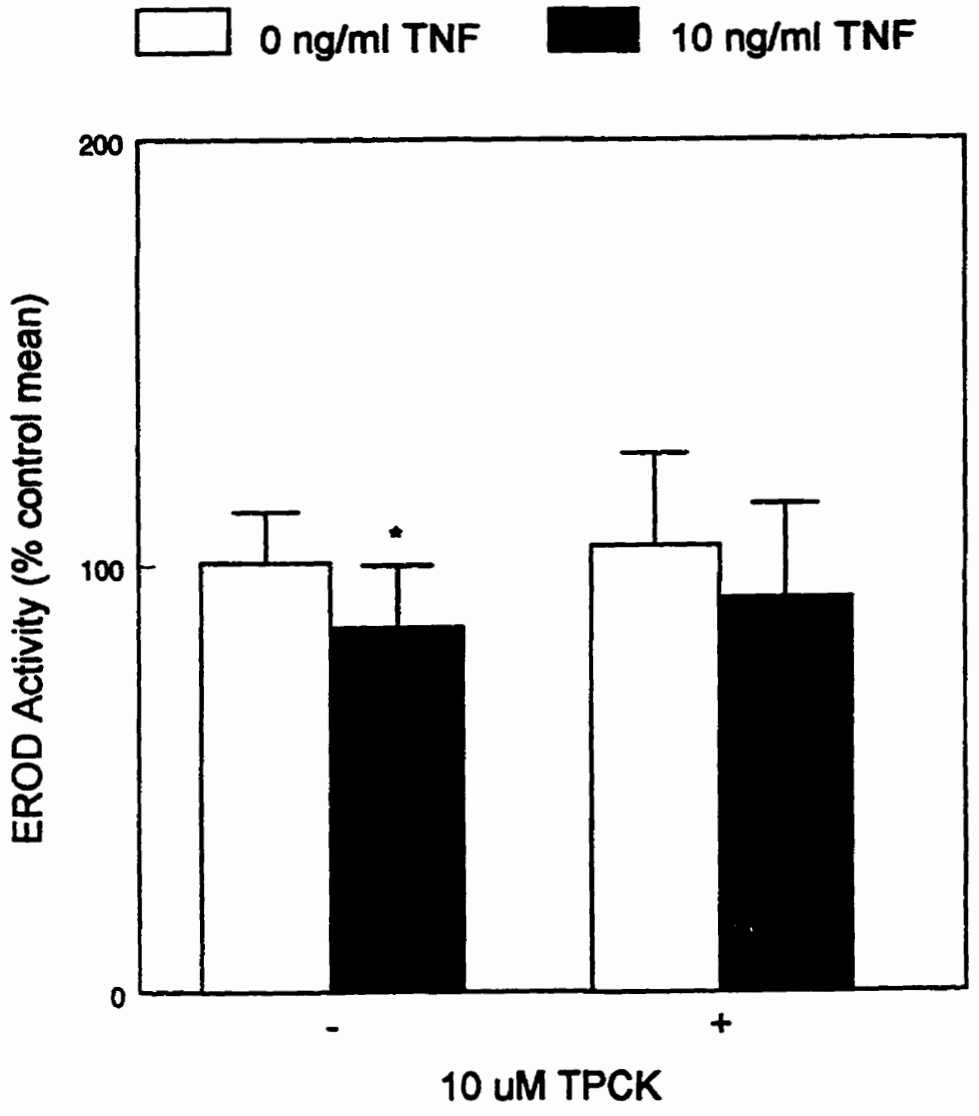


FIGURE 35

Effect of the NF-κB inhibitor, N-tosyl-l-phenylalanine chloromethyl ketone (10uM) on CYP1A depression by TNF.
CYP1A was induced with BNF.
Mean of control was: 5.38 ± 2.85 pmol/mg/min.
n = 12, using 3 homogenous pools, each obtained from 4 to 8 mice.
* significant difference from control (adjacent open bar). $p < 0.05$.

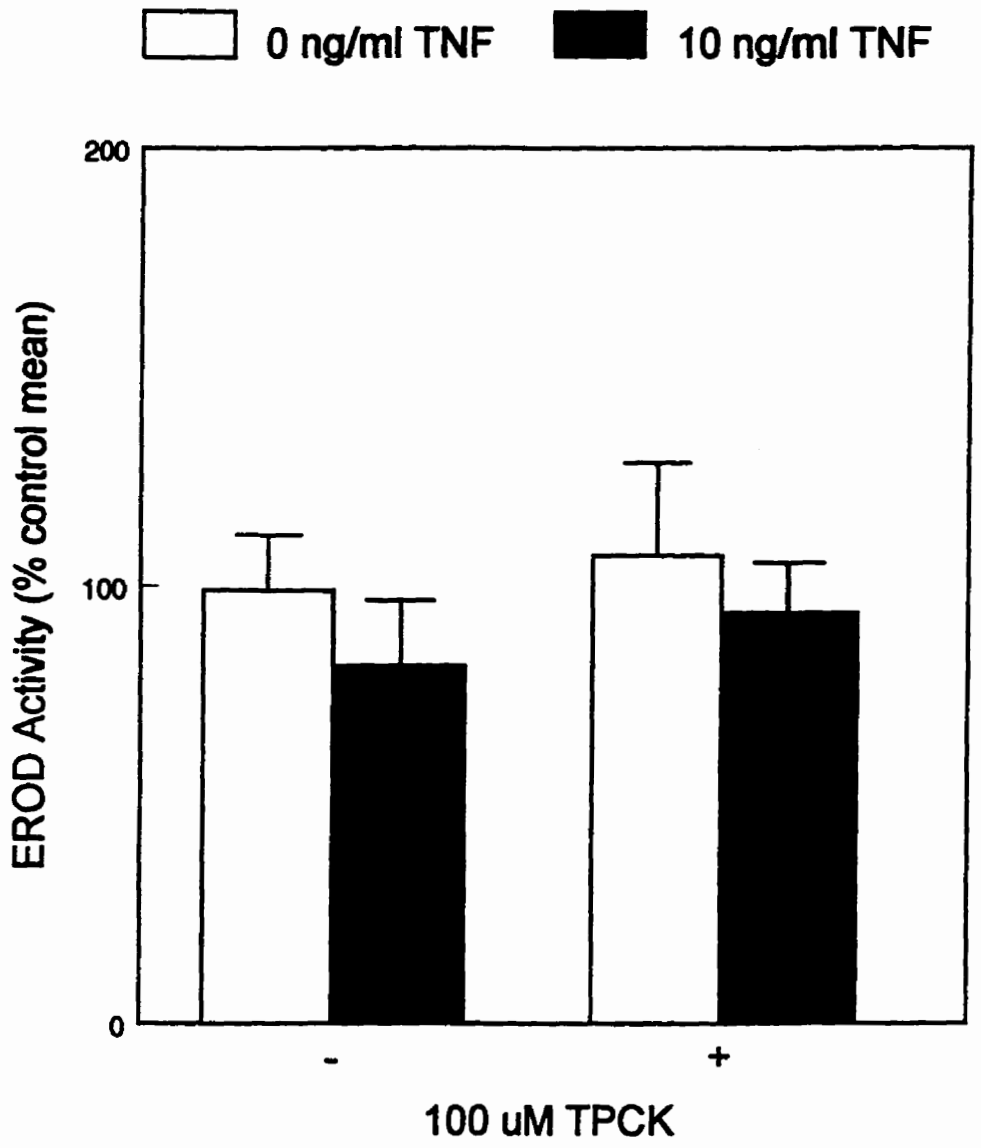


FIGURE 36

Effect of the NF-κB inhibitor, N-tosyl-l-phenylalanine chloromethyl ketone (100 uM) on CYP1A depression by TNF.
CYP1A was induced with BNF.
Mean of control was: 3.22 ± 0.20 pmol/mg/min.
n = 8, using 2 homogenous pools, each obtained from 4 to 8 mice.

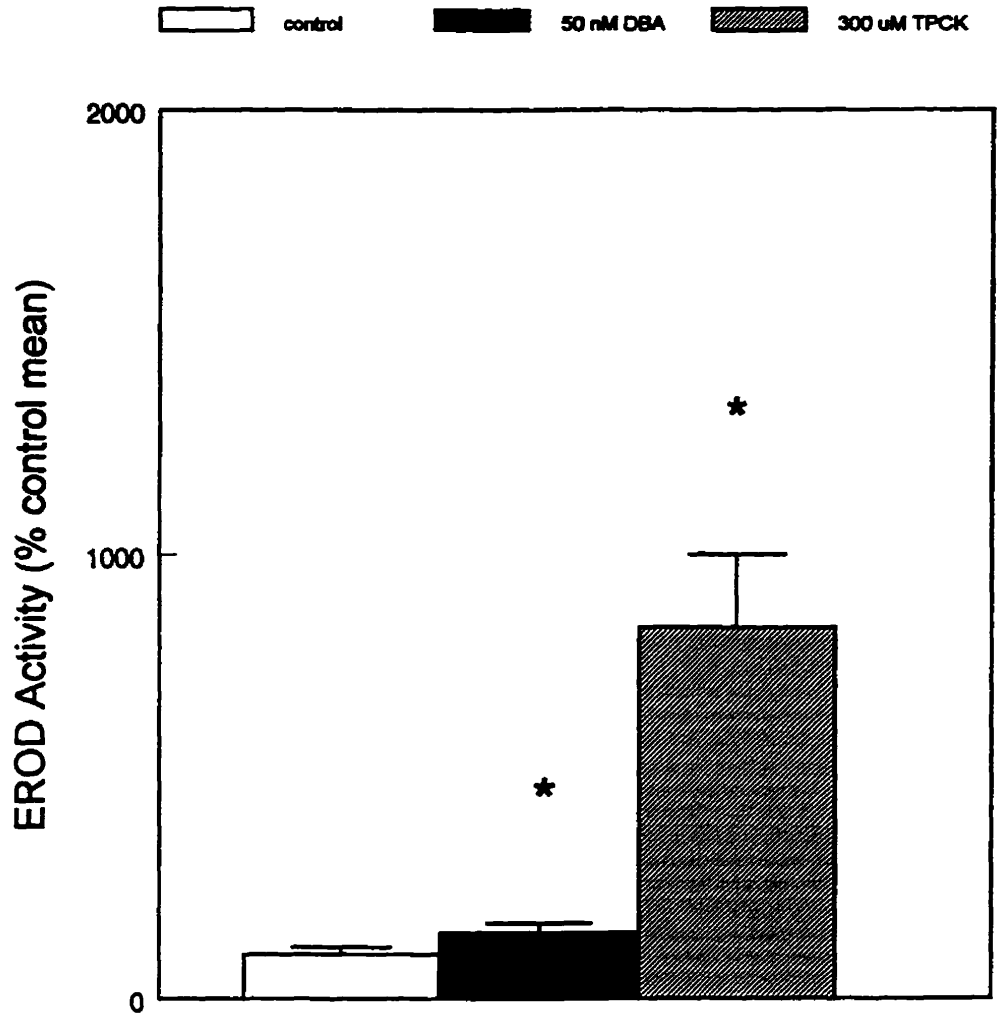


FIGURE 37

Effect of the NF-κB inhibitor, N-tosyl-l-phenylalanine chloromethyl ketone (TPCK) (300 uM) on CYP1A activity.
CYP1A activity was not induced.
Mean of control was: 7.23 pmol/mg/min.
n = 4, using 1 homogenous pool obtained from 4 to 8 mice.
* significant difference from control (open bar). $p < 0.05$.

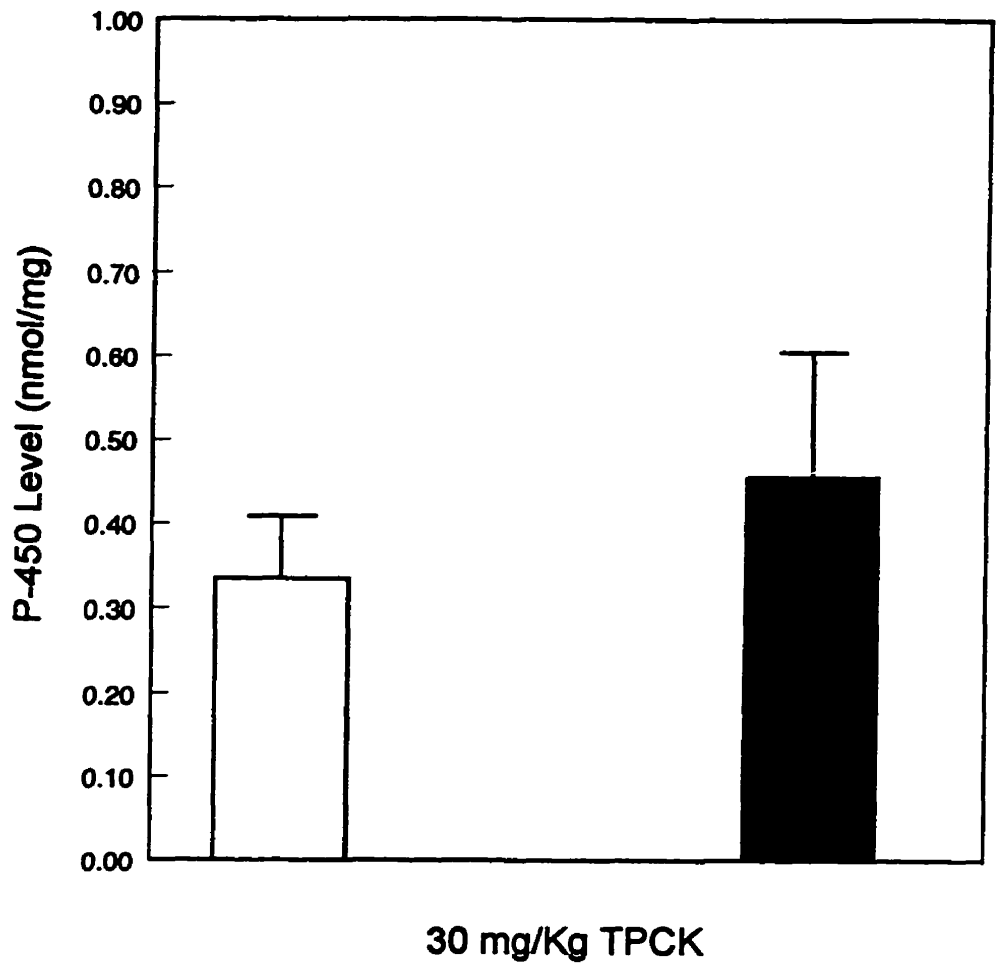


FIGURE 38

Effect of the NF-κB inhibitor, N-tosyl-l-phenylalanine chloromethyl ketone (TPCK) (30 mg/kg) on total cytochrome P-450 level *in vivo*.
Cytochrome P-450 level (nmol/mg protein), as determined by carbon monoxide binding spectrum,was found in microsomes prepared from mice which had been administered vehicle (open bar), or 30 mg/kg TPCK in ethanol (solid bar) IP.
n = 4 for each group.
Student t-test. $p < 0.05$.

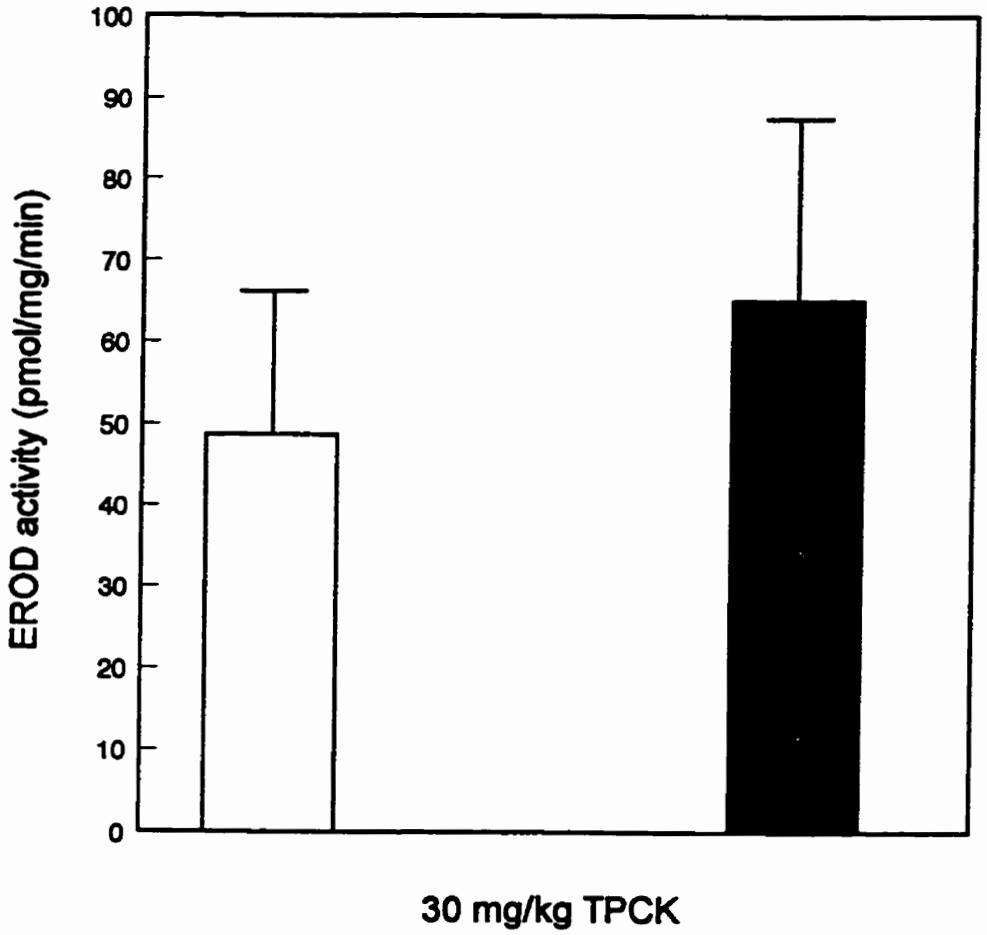


FIGURE 39

Effect of the NF-κB inhibitor, N-tosyl-l-phenylalanine chloromethyl ketone (TPCK) (30 mg/kg) on CYP1A activity *in vivo*.
CYP1A (pmol/mg/min), as determined by ethoxyresorufin-o-demethylase activity, was found in microsomes prepared from mice which had been administered vehicle, or 30 mg/kg TPCK in ethanol IP.
n = 4 for each group.
Student t-test. $p < 0.05$.

4.0 DISCUSSION

The objective of this project was to characterize the process of cytokine mediated decreases in P-4501A activity in a model of host defense activation in terms of a) time course, b) responsiveness to iNOS inhibitors, c) responsiveness to protein synthesis inhibitors, and d) responsiveness to NF-κB inhibitors. The main hypothesis was that TNF and possibly other cytokines, act at the hepatocyte to activate the transcription factor NF-κB. This factor then translocates to the nucleus, where it signals an increase in transcription of a variety of genes, some of which code for protein mediators which may be key to the depression of cytochrome P-450, including those for the inducible form of nitric oxide synthase. This increase in iNOS leads to a subsequent increase in nitric oxide which may act directly on cytochrome P-450, to destabilize either the heme group or the apocytochrome. Alternatively the NO may act to prolong the NF-κB activation, allowing for the prolonged production of proteins, some of which may have actions on the transcription and stability of apocytochrome mRNA.

Adequate separation of Kupffer cells from the hepatocytes was essential to this study, as factors released from these monocytes would affect the results. One of the major advantages in the use of mouse hepatocytes, is that the Kupffer cells can be separated from the hepatocytes with simple centrifugation, where as in rats, the similarities in size and densities of these two cell types make them difficult to separate by this procedure. Kupffer cells, being monocytes, are able to phagocytose small particles, and this feature was used to identify the cells in culture. Using fluorescent latex beads, it was

demonstrated that the hepatocyte cultures contained few contaminating Kupffer cells. The concern with contamination is that the cytokines or inhibitors added to hepatocyte cultures might be acting through a pathway involving Kupffer cells. This potentially confounding factor was not a problem in the current studies because of the homogeneity of the hepatocyte preparation, as determined with fluorescent latex beads. In addition LPS, even in very high doses, did not depress CYP1A activity when added directly to hepatocytes (fig. 16). When LPS was incubated with Kupffer cells, there was a release of substances which produced a depression in CYP1A activity when added to hepatocytes (fig. 16). If there had been contaminating Kupffer cells in the hepatocyte cultures, it would be predicted that the direct addition of LPS would activate these Kupffer cells, and that this would cause a depression in CYP1A activity. From these results, it appears that the amount of Kupffer cell contamination was minimal, and was certainly not sufficient to have an impact on the results presented in this thesis.

In primary hepatocyte cultures, cytochrome P-450 levels decrease by approximately 80% within 24 hours (Renton et al, 1978). These cells do not experience a further depression in cytochrome P-450 in response to interferon. However, primary cells whose cytochrome P-450 levels have been chemically induced, do respond to interferon. Similar reports for other cytokines, suggested that CYP1A levels should be chemically induced in order to then examine the effects of cytokines in cultured hepatocytes. Two methods of inducing the hepatocytes were chosen for the work presented in this thesis.

One method involved inducing CYP1A in vivo, prior to the isolation of the hepatocytes. The chemical inducer BNF was administered to the mice over the three day

period prior to the isolation of cells. The major advantage of this method is that in vivo induction minimized culture times, which was desirable as dedifferentiation occurs over time in primary cultured hepatocytes. Other concerns with longer culture times include an increased risk of infection, cell responses associated with medium changes, decreased viability, and time efficiency. This in vivo method of CYP1A induction also offered the advantage of reducing the number of chemicals added to the cultured cells. The disadvantages of this method of induction is that the time in the animal holding facilities is increased, and the separate set of mice are required to obtain the Kupffer cells, thus increasing the cost. Additionally, the magnitude of the induction was more variable with this method than the induction achieved using a chemical added directly to the cultured cells. Finally, this method did not allow for the use of uninduced control cells.

As previously described, the other method used to induce CYP1A was to add a chemical inducer (DBA) directly to the cultured cells. This method produced more cosistent induction, and it allowed for the same animals to be used for Kupffer cell isolation as were used for the hepatocyte isolation, which was convenient, cost effective, and consistent. The disadvantages are that the cells remain in culture for a longer period of time, and that they are exposed to additional compounds, increasing the risk of contamination, and adding to the complexity of the system.

As shown in figure 16, the addition of LPS directly to hepatocyte cultures did not affect CYP1A activity, but the effect of adding medium from LPS treated Kupffer cells was to produce a strong depression of CYP1A activity. It is known that monocytes can be stimulated by the immunostimulant dextran sulfate to release cytokines and other

mediators, and that the medium containing these factors will cause a depression in cytochrome P-450 activities and levels when added to isolated hepatocytes (Renton, 1983). The purpose of these experiments was to establish a model to look at potential inhibitors of this effect. However, in our experiments, the response lacked reproducibility, possibly because of variations in the Kupffer cell yield between experiments. In addition, the identification of the active components of the medium would be difficult, costly and time consuming. Direct stimulation with a single cytokine (TNF) was therefore chosen to conduct the inhibitor experiments in order to simplify the system.

Treatment of the hepatocytes with medium from untreated Kupffer cells (figure 16, bar 3) produced a variable response in CYP1A activity. In many cases, it had little effect, or a trend towards a slight, but not statistically significant induction of CYP1A, as shown in fig.13, 14, 15, and 16. In other cases, as in fig. 12, the medium from the untreated Kupffer cells caused a depression of CYP1A activity. This depression in activity could be explained by activation of the control Kupffer cells by some factor or factors other than LPS, such as physical stress, or contamination, or possibly by the transfer of some non-adhering Kupffer cells into the hepatocyte plate. In many cases (data not shown), the depression of CYP1A activity was as great in the hepatocytes treated with medium from untreated Kupffer cells as it was in the hepatocytes from LPS treated Kupffer cells. This contributed to the preference of using the individual cytokine, TNF, over the use of conditioned medium. Possible explanations for the slight induction seen in response to the medium from untreated Kupffer cells (fig. 13, 14, 15, and 16) are less obvious, and may represent experimental error, or a change in the composition of the medium taken from

Kupffer cells that leads to induction of CYP1A activity.

LPS is a compound with a large molecular weight ranging from 1-4 million daltons. Figure 14 and 15 are representative experiments from using filtered conditioned medium. Filtering the medium did not change the effectiveness of the medium from LPS treated Kupffer cells. This demonstrates that the action of the medium is due to some small particle, as opposed to an alteration of the LPS molecule by the Kupffer cell into an active form, or an action of LPS on Kupffer cell adherence allowing the LPS treated Kupffer cells to be transferred into the hepatocyte fraction. The response of hepatocytes to medium from LPS treated Kupffer cells are consistent with those found by other groups.

The effect of the mycotoxin, deoxynivalenol (DON) was examined because of its reported stimulation of cytokine production (Aconza-Olivera et al, 1995, Dong et al, 1994). DON is found on wheat and corn, and was suspected as a cause for recent illness seen in horses and the humans handling their feed. It has been found that administration of DON produces elevated levels of the mRNA of several cytokines, including IFN, IL-1, IL-6, and TNF. Each of these cytokines are known to cause a depression of cytochrome P-450. This elevation in cytokine mRNA was interpreted as an increase in cytokine levels, and therefore, DON might be expected to produce a decrease in cytochrome P-450 dependent metabolism. In this study, oral administration of DON did not depress cytochrome P-450 levels (fig. 17), or CYP1A activity (fig 18) in the liver. There was even a slight increase in CYP1A activity after oral administration of DON. There was also no effect of DON when incubated with hepatocytes, or when medium, obtained from

Kupffer cells which had been treated with DON was incubated with hepatocytes. Medium taken from Kupffer cells treated with LPS was able to depress CYP1A activity, indicating that there were sufficient responsive Kupffer cells to produce the amount of cytokines needed to depress CYP1A activity. One explanation for the lack of effect of DON may be that it did not produce an elevation in cytokine levels despite its reported effects on cytokine mRNA. DON also possesses actions as a protein synthesis inhibitor (Dong et al, 1994, Petska et al, 1987). The evidence for its ability to elevate cytokine levels has been based on its ability to elevate their mRNA. It is possible that the actions as a protein synthesis inhibitor prevent the translation of this mRNA into increased protein. Another possible explanation for the failure of the mycotoxin to depress CYP1A activity may be that it has its own effect inducing on cytochrome P-450 which competes with the cytokines. Again, this may relate to its protein synthesis inhibition. Cytokine mediated depression of cytochrome P-450 requires the synthesis of an intermediate protein, and the mycotoxin may have produced the elevated levels of cytokines, but may have inhibited the production of a critical intermediate protein within the hepatocyte through its action as a protein synthesis inhibitor. This inhibition of protein synthesis may also be a part of the effect DON had on CYP1A activity in vivo, as protein synthesis inhibitors have been reported to superinduce m RNA fro CYP1A1 (Nemoto et al, 1991).

This possibility could be explored by performing an ELISA on the serum of animals treated with DON, or on the medium from Kupffer cells treated with DON to determine if there in fact was an elevation of cytokine levels. Another approach to this problem would be to add medium from Kupffer cells treated with LPS, or to add a single

cytokine known to depress cytochrome P-450 in addition to the DON. If these stimuli failed to produce a depression in cytochrome P-450 in the presence of DON, it could be conclude that DON is able to block the action of cytokines on CYP1A activity. However, if these stimuli were able to depress cytochrome P-450 in the presence of DON, it would support the theory that DON does not produce sufficient quantities of cytokines to affect cytochrome P-450.

Direct cytokine stimulation offered the advantage of being more consistent between experiments. In addition, it was desirable to have a specific stimulus, as opposed to an undefined mixture of components. The time course for TNF's ability to produce depression of CYP1A in this study is consistent with that found in mice injected with TNF in a study by Ghezzi et al. In that study, there was a rapid response, significant by 8 hours after administration, and continuing until 24 hours. By 48 hours, the levels had returned to normal. This present study found that there was a significant depression of CYP1A activity at 6 hours, continuing until 24 hours of treatment with TNF (fig. 16). Ghezzi et al produced a depression in ethoxycoumarin deethylase activity of approximately 30% at 24 hours, with a dose of TNF (5×10^4 U/mouse i.v.) that produced the maximum possible response. In the present study, TNF was demonstrated to depress CYP1A activity by 32% from control at 24 hours (fig. 16). The results obtained in this cell culture system are consistent with the data from in vivo assays (Gezzi et al, 1986). The time course for the decrease in CYP1A activity is consistent with the theory that TNF signals the production of NOS2, which then releases nitric oxide that can act to functionally inhibit cytochrome P-450. The results from Western blot analysis are also consistent with a

functional inhibition, as 24 hour stimulation with TNF produced only a slight depression in the level of CYP1A protein. The findings from this Western blot differ from the literature in general, and the reproduciblity of this result has not been established, therefore it cannot be ruled out that this represents experimental error, however the finding is consistent with previous work by Paton (1996), where 10 ng/ml TNF-α had no effect on CYP1A protein level when incubated with Hepa1 cells, despite a reduction in CYP1A activity. One possible explanation for this is that the decrease in activity is due to a functional block of the enzyme by nitric oxide binding to the heme group. Another possibility is that the depression in CYP1A activity seen in response to TNF is due to a depression in the level of CYP1A2, which would not be detected in the Western immunoblot.

The existence of an intermediate protein mediating the effect of cytokines on cytochrome P-450 was established by the observation that the depression of cytochrome P-450 by in vivo administration of recombinant interferon was blocked in the presence of Actinomycin D or Puromycin, inhibitors of protein synthesis (Renton et al, 1990). Interferon added directly to hepatocytes depresses cytochrome P-450, suggesting that the intermediate protein must be synthesized within the hepatocyte itself (Monshower et al, 1995). There was a difference in the response to protein synthesis inhibitors of hepatocytes in which CYP1A was induced by adding DBA to the plated hepatocytes compared with hepatocytes in which CYP1A had been induced prior to isolation by the IP administration of BNF to the mice. In the DBA induced cells, the administration of the protein synthesis inhibitors alone caused a significant reduction in CYP1A activity relative to the untreated cells (fig. 23 and fig. 25). In the cells from BNF treated mice, this

reduction did not occur, and Actinomycin D tended to produce a slight, but not significant increase in the CYP1A activity (fig. 24). TNF-α failed to depress CYP1A in the presence of the protein synthesis inhibitors relative to the inhibitors alone, indicating that protein synthesis within the hepatocyte is required to produce this depression in CYP1A activity. From these experiments, it appears that induction of CYP1A prior to isolation of cells provides a model that more closely resembles the in vivo situation. One possible explaination for the differences seen between the cells induced *in vitro* versus *in vivo*, is that in the DBA treated cells, the inducer may still be present, and CYP1A synthesis may be ongoing. The depressing effect of the protein synthesis inhibitors may have to do with an interuption of that process. In the cells from BNF treated mice, the induction is more likely to be complete, and thus the protein synthesis inhibitors alone have less of an effect.

Nitric oxide synthase is one possibile candidate for the intermediate protein. To investigate this, it was confirmed that the nitric oxide generating compound SNP was able to depress CYP1A activity in this culture system. The depression in activity was seen over the initial 30 minutes, which is the earliest time point which can be measured using this technique. The speed with which SNP elicits this effect is not consistent with a mechanism involving a decrease in the production and stability of mRNA, or a decrease in the synthesis of the apoprotein. This response is more consistent with a functional inhibition of the CYP1A due to NO binding to the heme portion of the enzyme. This is supported by Western blot analysis of the protein levels which did not show a decrease in the amount of CYP1A in response to SNP, even at the 24 hour time point, despite a depression in the CYP1A activity.

Controversy exists over the involvement of nitric oxide in the depression of cytochrome P-450. Nitric oxide synthase inhibitors have been reported to block the depression of cytochrome P-450 by cytokines both *in vivo*, and *in vitro*, however, other studies suggest that the production of nitric oxide is not required for the depression in enzyme activity. NIL and NIO, selective inhibitors of NOS2, have not previously been used in the study of the effect of cytokines on cytochrome P-450. As expected, NIL (100 μM) was able to prevent any depression in CYP1A activity in response to TNF. NNA, a non-selective NOS inhibitor, NIO, and NIL (10 μM) reduced the extent of the depression in CYP1A after TNF stimulation to below a significant difference, but the effect is more modest. The depression of CYP1A activity in the presence of 100 μM NNA and in the presence of 100 μM NIL was unexpected. The reason for this depression is unclear, and is difficult to rationalize. If there were a basal level of nitric oxide production, it would be expected to be exerting an inhibitory tone on CYP1A activity, and the addition of an inhibitor would lead to a removal of this tone, and an increase in CYP1A activity. It was not obviously due to cytotoxicity as determined by visual observation, trypan blue, or protein content. Visual observation and trypan blue exclusion accompanied by visual observation may not be sufficiently sensitive. Cell death would likely be detected in a change in total protein, as dead cells would be less adherent, and therefore would be removed from the plate during the washing step which preceded the scraping of the plates. As all of the plates began with approximately the same amount of protein, plates with conditions causing cell death might show a decrease in protein. This was also not the case, however, this may also not be sufficiently sensitive to detect small changes in cell

viability. Another possible cause of the effect of the nitric oxide synthase inhibitor relates to the fact that nitric oxide decreases total hepatic protein synthesis. If small amounts of nitric oxide present under these control conditions were inhibiting the synthesis of a protein whose function is to inhibit CYP1A levels or activity, then the NOS2 inhibitor would remove that protein synthesis inhibition, allowing the production of this inhibitory protein, and thereby lead to the decrease in CYP1A activity. The existence of such an inhibitory protein expressed under control conditions has been proposed by others, as part of the explanation for the increase in cytochrome P-450 seen under some conditions after exposure to protein synthesis inhibitors (Nemoto et al, 1991). The sensitivity of such a protein to small amounts of nitric oxide has not been demonstrated, nor has the presence of small amounts of nitric oxide under the present conditions. Highly sensitive techniques for the detection of nitric oxide might be able to determine if any nitric oxide is present under these control conditions. NOS2 can be induced by factors other than cytokines, such as physical stress, so it is not implausible that there may be some NO released during the isolation procedure. If the use of an antibody to completely inhibit the nitric oxide synthase also led to such a depression in CYP1A activity, it would support a theory that low levels of nitric oxide present in the control conditions were involved with elevating CYP1A activity. In the presence of such an antibody, very small amounts of a nitric oxide donor could be added to see if a concentration exists where CYP1A activity is elevated.

The results found here are in agreement with an involvement of nitric oxide in the cytokine mediated depression of CYP1A activity, as the potent and selective inhibitor was successful at preventing a depression in CYP1A activity in response to TNF.

The possible involvement of the transcription factor NF-κB in this process was suggested by a number of lines of evidence, as discuused in section 1.6. Two inhibitors of NF-κB were used to determine if activation of this transcription factor is a requirement for the depression in CYP1A in response to TNF. The inhibitor BTEE was effective at blocking the depression in CYP1A activity produced by TNF, however the large standard deviations associated with the addition of the drug make it difficult to draw any conclusions from these results. The inhibitor TPCK at 10 μM or 100 μM had little or no effect on the depression of CYP1A activity. Surprisingly, at 300 μM, TPCK had a very strong inducing effect on CYP1A activity. This occurred even in the absence of any inducer.

The results are not conclusive, but the results with BTEE suggest that there could be a role of NF-κB in the depression of CYP1A activity. NF-κB activation has been identified as a necessary step for the expression of the NOS2. As NO has been identified as a mediator of the depression of cytochrome P-450, the activation of the transcription factor should also be a

part of the pathway leading to the depression of CYP1A. If the NF-κB inhibitors had inhibited NF-κB, but had failed to inhibit the depression in CYP1A, it would be demonstrated that either NOS2 expression is not dependent on NF-κB, or that NO is not required for the inhibition of CYP1A activity. It is difficult to confirm the activation state of the transcription factor, as the antibodies available are developed in mice. Rat cells were isolated in an effort to clarify this problem, however, rat hepatocytes are more difficult to separate from Kupffer cells, and the influence of contaminating Kupffer cells is

a concern for these cytokine experiments. In addition, there was background fluorescence, and the activation in response to TNF was difficult to distinguish from control cells. Mouse antibodies can be used in the mouse cells, if they are prelabeled with a fluorescent component before addition to the cells, however, the time required to develop this technique would go beyond the completion of the project. Other NF-κB inhibitors are available, and the effect of these on CYP1A activity, and the depression of that activity in response to cytokines may be worth pursuing.

The direct CYP1A inducing effect of TPCK at 300 μM is interesting if for no other reason than that this is such an effective inducer. The phenyl alanine group of this drug could be argued as a potential site for it to be acting as a simple chemical inducer, although this seems unlikely, as phenyl alanine is an endogenously expressed compound. On the other hand the induction of CYP1A may be an effect of inhibition of NF-κB. NF-κB normally exists in an inactive form in the hepatocyte (Freedman et al, 1992). During the initial three hours of adherence to a plate, NF-κB becomes active, and remains that way for a prolonged period of time. The levels of cytochrome P-450 drop by 80% during the initial 24 hours after plating (Freedmean et al, 1992). The induction of cytochrome P-450 in response to TPCK may represent an inhibition of the decrease in cytochrome P-450 seen after plating. This hypothesis is supported by the in vivo results where 30 mg/kg TPCK failed to elevate cytochrome P-450 levels, or CYP1A activity after 24 hours (fig. 38, fig. 39). If the CYP1A induction in cultured hepatocytes in response to TPCK is related to its inhibition of NF-κB activation, it is not surprising that it had no effect in vivo, where NF-κB is present in an inactive state. This finding is consistent with a

hypothesis that NF-κB activation is involved in the decrease in cytochrome P-450 seen after plating. There are many questions remaining to be answered related to this finding. This issue could be examined in a variety of ways, for example, examining the effect of the NF-κB inhibitor on constitutive forms of cytochrome P-450 depressed during culture, but insensitive to chemical inducers would answer the question of whether TPCK is acting as a chemical inducer. Inhibiting the transcription factor with an antibody and looking at the effect on cytochrome P-450 in culture, to see if it induced CYP1A activity, or adding NF-κB inhibitors to hepatocytes cultured in a manner that conserves its cytochrome P-450 content, such as collagen gel matrices, or rotating culture flasks, to see if there is an induction of cytochrome P-450 under these conditions.

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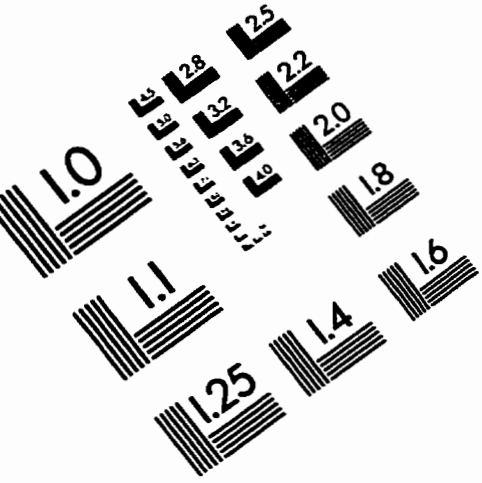
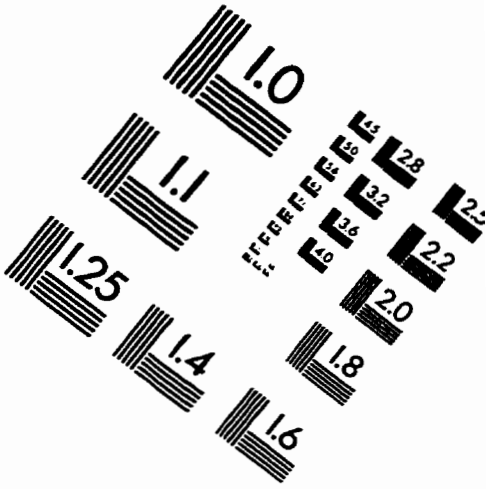
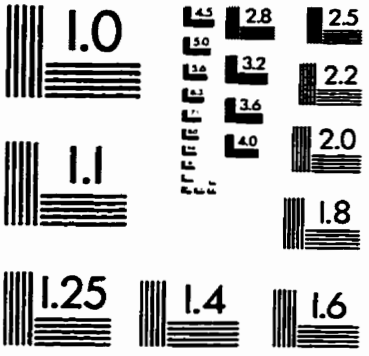
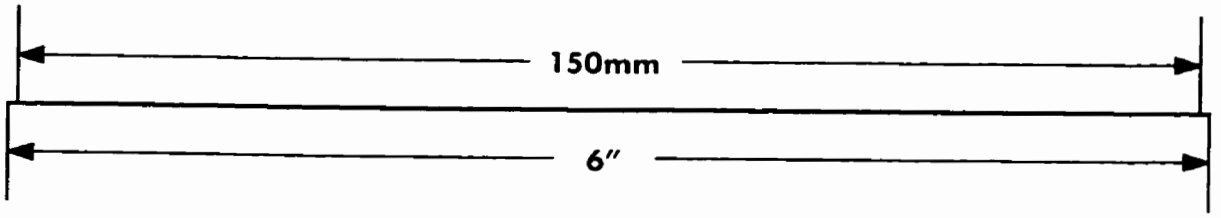
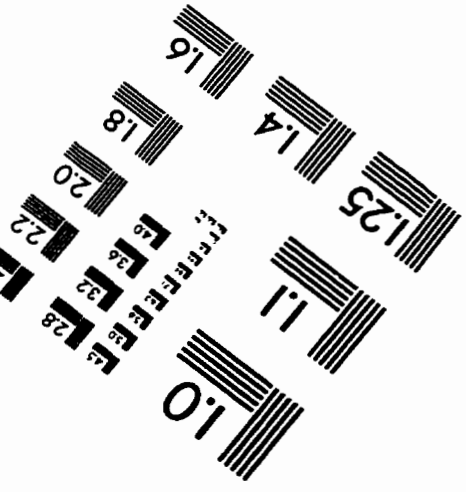
IMAGE EVALUATION TEST TARGET (QA-3)

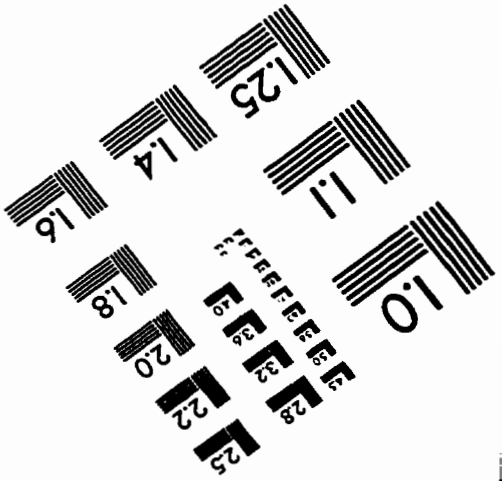












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