Effect of cytochrome P-450 1A1 induction on oxidative damage in the brain and liver

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Effect of Cytochrome P-450 1A1 Induction on
Oxidative Damage in the Brain and Liver

by

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B.S., Pharmacy, Jiangxi College of Chinese Medicine

Presented in partial fulfillment of the requirements for the
Degree of Master of Science
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1998

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Chairman, Board of Examiners
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8-12-98
Date
Polycyclic and halogenated aromatic hydrocarbons (PAHs and HAHs) are widespread and persistent environmental contaminants. Most of their toxicological effects are mediated through activation of the aryl hydrocarbon receptor (AhR), which leads to transcriptional activation of cytochrome P-450 1A1 and 1A2 (CYP 1A1/2) genes. Induction of CYP 1A1/2 increases drug metabolism and enhances the generation of reactive oxygen species (ROS) in vivo. Oxidative stress is an important mediator of various neurological and hepatic disorders. In the brain, oxidative damage can alter key components of glutamate homeostasis, such as glutamine synthetase (GS). This change can generate abnormally elevated extracellular glutamate, and lead to excitotoxicity.

PAHs and HAHs have been shown to induce CYP 1A1 in rat brain. However, whether the induction of CYP 1A1 can cause oxidative damage or interact with other pathologies in the central nervous system has not been reported.

In the present study, we investigated the effect of CYP 1A1 induction on oxidative status in the brain and liver. We demonstrated that induction of CYP 1A1 by 3-methylcholanthrene (3-MC) did not oxidize proteins or lipids, or decrease GS activity in rat brain and liver. Furthermore, the induction of CYP 1A1 in the brain and liver did not enhance the extent of lipid peroxidation in either tissue following oxidative challenge. If increased CYP 1A1 activity in the brain and liver following 3-MC treatment leads to increased ROS formation, the increase is insufficient to overwhelm the endogenous antioxidant defense system, produce detectable oxidative damage, and alter glutamate homeostasis.
Abbreviations

AhR: aryl hydrocarbon receptor
AhREs: aryl hydrocarbon-response elements
Arnt: aryl hydrocarbon receptor nuclear translocator
CNS: central nervous system
CYP: cytochrome P-450
CYP 1A1: cytochrome P-450 1A1
CYP 1A2: cytochrome P-450 1A2
CYP 2E1: cytochrome P-450 2E1
EROD: ethoxyresorufin O-deethylase
GS: glutamine synthetase
GSH: glutathione
GST: glutathione S-transferase
HAHs: halogenated aromatic hydrocarbons
H₂O₂: hydrogen peroxide
Hsp90: heat shock protein 90
3-MC: 3-methylcholanthrene
α-NF: α-naphthoflavone, CYP 1A1 inhibitor
β-NF: β-naphthoflavone, CYP 1A1 inducer
O₂⁻: superoxide anion
OH: hydroxyl radical
PAHs: polycyclic aromatic hydrocarbons
ROS: reactive oxygen species
TBARS: thiobarbituric acid-reactive substance
TCDD: 2, 3, 7, 8-tetrachloro dibenzo-p-dioxin
UDP-GT: UDP glucuronosyltransferase
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Introduction:

Polycyclic and Halogenated Aromatic Hydrocarbons:

Polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) are widely distributed environmental toxins. Major sources of PAH exposure include cigarette smoke, smoked food products, and air pollution (Costa, 1998). The HAH family includes polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs). HAHs are mainly industrial contaminants. PCBs are used in heat transfer fluid. PCDDs and PCDFs are byproducts in the synthesis of PCB, phenoxyherbicides, and chlorinated phenols. PCDDs are also produced by paper mills, forest fires, and automobile exhaust (Costa, 1998).

In spite of wide exposure, the levels of PAHs in body tissues are normally unmeasurable (Wolff, et al., 1996). PAHs are typically short lived in the body and are metabolized by several cytochrome P-450s (CYPs) to electrophilic derivatives. The principle enzymes involved in PAH metabolism are the 1A1 and 1A2 subtypes of cytochrome P-450 (CYP 1A1/2). Cytochrome P-450 2B1 (CYP 2B1) also plays a role in PAH metabolism (Hankinson, 1995). In contrast, HAHs are relatively resistant to metabolism.
and more likely to build up in liver and adipose tissue upon exposure (Hankinson, 1995; Abraham, et.al., 1988). The apparent half life for 2, 3, 7, 8-tetrachloro dibenzo-p-dioxin (TCDD) is up to several weeks in rodents (Rose, et.al., 1976), and is even longer in primates (Krowke, 1986; Poiger and Schlater, 1986).

Most of the toxic effects for PAHs and HAHs are caused by activation of the aryl hydrocarbon receptor (AhR) (Hankinson, 1995; Dogra, et.al., 1998). Both PAHs and HAHs can transcriptionally induce biotransforming phase I and conjugating phase II metabolic enzymes (Hankinson, 1995; Poellinger, 1995). The induction of these enzymes significantly changes the metabolism for exogenous drugs and toxicants (Pitot III, 1995; Parkinson, 1995). The attack of the electrophilic metabolites of PAHs on DNA leads to mutation and tumor formation. Long term exposure of PAHs and HAHs have been reported to cause sarcoma, hepatic cancer, and breast cancer in rodents (Pitot III, 1995; Kociba, 1982). In addition, toxicities for PAHs and HAHs also include reproductive problems (Tomas, 1995; Brouwer, et.al., 1995), endocrine dysfunction (Capen, 1995), developmental retardation (Brouwer, et.al., 1995), and cardiovascular pathology (Ramos, et.al., 1995). A considerable body of research in the last fifteen years has established specific
biological cascades involved in the effects of PAHs and HAHs. PAHs and HAHs alter intracellular Ca^{2+} homeostasis (Costa, 1998), transcriptionally induce specific genes through the Aryl hydrocarbon receptor (AhR) (Nobert and Gonzalez, 1987; Quattrochi, 1989; Landers, 1991), and induce other genes not associated with AhR (Okey, et.al., 1994).

Figure 1. Basic scheme for the transcriptional activation of CYP 1A1/2 genes by PAHs and HAHs. TCDD, tetrachloro dibenzo-p-dioxin; MC, 3- methylcholanthrene; AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator protein; AhREs, aryl hydrocarbon receptor-responsive element; 1A1, CYP 1A1; 1A2, CYP 1A2. (Okey, et.al., 1994)

PAHs and HAHs bind to a cytosolic protein, AhR, when they enter into the cells. Latent AhR is complexed with heat shock protein 90 (Hsp90).

Following binding, AhR is activated and the complex is transported into the
Following binding, AhR is activated and the complex is transported into the nucleus. Hsp90 is then released from the complex and the transformed complex dimerizes with the aryl hydrocarbon nuclear translocator (Arnt). The AhR-Arnt heterodimer binds to specific DNA sequences, known as the aryl hydrocarbon-response elements (AhREs), and induces the transcription of numerous metabolic enzymes, cytokines and growth factors (Okey, et.al., 1994). The AhRE sequence, located upstream from the activated gene, functions as an enhancer region (Dogra, et.al., 1998). Genes encoding both phase I and phase II enzymes can be transcriptionally induced by PAHs through this AhR-dependent mechanism. These genes include the phase I enzymes CYP 1A1/2 and aldehyde dehydrogenase (ALDH), and the phase II enzyme glutathione S-transferase (GST) (Okey, et.al., 1994).

TCDD, 3-Methylcholanthrene (3-MC), and β-naphthoflavone (β-NF) are potent CYP 1A1 inducers. These compounds can induce CYP 1A1 in the liver and also extrahepatic tissues (Juchau, et.al., 1979; Schilter and Omiecinski, 1994; Unkila, et.al., 1993). For example, 3-MC (5 injections of 110 μmol/Kg) induces CYP 1A1 in both neurons and glial cells one day after treatment (Dhawan, A., 1990).
Cytochrome P-450:

Cytochrome P-450 (CYP) is a group of NADPH-dependent, oxidative enzymes capable of metabolizing a number of xenobiotics and endogenous compounds (Gonzalez, 1991). All CYPs are hemoproteins with a cysteine-thiolate ligand binding to heme iron (Gonzalez, 1991). CYPs can be classified in two general classes based on their means for obtaining electrons from NADPH and, in addition, their intracellular location in eukaryotes (Gonzalez, 1991). Mitochondrial CYPs, such as CYP 11A (side-chain cleavage cytochrome P-450) and CYP 17, are found exclusively in the inner mitochondrial membrane and are involved in steroid biosynthesis. They receive electrons via an iron-sulfur protein from flavin adenine dinucleotide (FAD)-containing enzyme (Gonzalez, 1991). The second class of CYPs is bound to microsomal membranes, and mainly involved in metabolism of foreign compounds. These microsomal CYPs obtain electrons via NADPH-cytochrome P-450 reductase, and cytochrome b₅ (Gonzalez, 1991). The microsomal CYPs are divided into the four gene families: CYP 1, 2, 3, and 4. All CYPs within a single gene family exhibit greater than 40% sequence homology, and CYPs within the same subfamily have more than 55% identity in sequences (Gonzalez, 1991). CYPs catalyze one-electron delivery.
oxidative reactions in vivo and in vitro, such as carbon hydroxylation, heteroatom release reactions, and oxidation of unsaturated bonds (Guengerich, 1991).

Multiple cytochrome P450 isozymes have been purified and characterized in different tissues in rodents and mammals. The earliest work was conducted on hepatic enzymes, due to the relatively high concentration of CYP and the significance of the liver in metabolism of various drugs and cholesterol. The expression, function, and regulation of isoforms of CYP differ among species. Human liver microsomes contain relatively high levels of CYP 1A2, but not CYP 1A1. In contrast to CYP 1A1, CYP 1A2 is not expressed in extrahepatic tissues (Parkinson, 1995). The most abundant CYP in mammalian and rodent liver is the CYP 3A gene family. CYPs 2A, 2C, 2D, 2E1, and 4A are also constitutively expressed in their liver (Gonzalez, 1991; Funae and Imaoka, 1991). The hepatic CYPs biotransform a wide range of xenobiotics and endogenous compounds (Parkinson, 1995). The expression and activity of different isoforms of CYP in the liver are regulated by numerous compounds (Parkinson, 1995; Dogra, et al., 1998). For example, ethanol is a potent CYP 2E1 inducer, while PAHs and HAHs
markedly induce CYP 1A1 in the liver. Inhibition or induction of CYPs affects the rate of xenobiotic transformation (Parkinson, 1995).

CYP enzymes have been detected in the brain. In untreated rats, the brain microsomal CYP concentration is approximately 1%-3% of that found in the liver (Warner, et al., 1988). The amount of CYP is unevenly distributed among brain regions. Major isoforms of hepatic CYP, such as CYP 3A2, 2C11, and 2A1, are either undetectable or only account for a small fraction of brain CYP (Warner, et al., 1991). CYP 1A1/2, 2B1, 3A1, and 2E1 are the main subtypes of CYP detected in the brain by immunohistochemical studies, western blotting, and RT-PCR (Warner, et al., 1991; Schilter and Omiecinski, 1994). CYP 1A1/2 have been detected in both neurons and glial cells (Kohler, et al., 1988). CYP 1A1/2 mRNA and enzyme are present in the striatum, hypothalamus, olfactory bulbs, and, to a lesser extent, in other regions of the brain (Kohler, et al., 1988; Scholter and Omiecinski, 1994). In contrast to the liver, exposure to the well-known hepatic CYP inducers, including PAHs, ethanol, and barbiturates, produces no more than a 150% increases in CYP activity in the rat brain, while liver CYP activity increases by an order of magnitude (Rouet, et al., 1981; Srivastava, et al., 1983).
In spite of relatively low concentrations in the brain, CYPs have very important physiological functions and toxicological consequences in the central nervous system (CNS). Brain CYPs metabolize drugs entering into brain, regulate intracellular cholesterol levels, activate signal transduction via the formation of arachidonic acid, and regulate vascular tone (Warner, et al., 1991). In addition, CYPs produce cytotoxic metabolites from industrial solvents and aromatic hydrocarbons in the brain. For example, bis (chloromethyl) ether, and acrylonitrile can cause glioma via a CYP 2E1 metabolite in experimental animals (Hopwell and Wright, et al., 1969; Maltony, et al., 1982). After ethanol treatment, increased generation of oxygen radicals in rat brain and astrocyte cultures can be partially inhibited by an antibody against CYP 2E1 (Bondy and Guo, 1994; Montoliu, 1995).

The CYP catalytic cycle involves the binding of the enzymes to substrate, followed by binding of molecular oxygen to the heme in CYP, activation of the complex to a reactive intermediate, and release of hydroxylated substrate (Park, et al., 1996). Autooxidation of the cytochrome P-450 complex produces superoxide (Kappus, 1991). The dismutation of superoxide anion leads to the formation of hydrogen peroxide. OH\(_8\)

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reactive oxygen radical, is produced by Fenton’s reaction when hydrogen peroxide reacts with reduced metalloproteins (Kappus, 1991).

Induction of CYPs by various substances enhances ROS generation in vivo and in vitro. The well known example is ethanol-induced oxidative stress in liver. After ethanol administration, an increase in ROS level and increase in lipid peroxidation occur. These effects can be effectively inhibited by antibody against CYP 2E1 (Higuchi, et.al., 1996; Lieber, 1997). Induction of CYP 1A1 by TCDD has been reported to cause oxidative DNA damage in hepatoma cells. The increased generation of 8-oxo-2’-deoxyguanosine, a biomarker of oxidative DNA damage, can be reduced by a CYP 1A1 inhibitor (Park, et.al., 1996).

Reactive Oxygen Species and Oxidative Stress:

Various kinds of neurological disorders and hepatic injuries involve oxidative stress. These include stroke, Alzheimer’s disease, Amyotrophic Lateral Sclerosis, Parkinson Disease, liver fibrogenesis, and alcoholic liver (Wilson, 1997; Markesbery, 1997; Jenner and Olanow, 1996; Liu, 1996; Lieber, 1997). In addition, oxidative stress plays a role in various age-associated diseases, including atherosclerosis, neoplastic diseases, diabetes, and chronic inflammatory diseases (Stohs, 1995).
Reactive oxygen species (ROS) are generated as intermediates during the reduction of oxygen to water. ROS include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$), singlet oxygen (\(^{1}\)O$_2$) and peroxynitrite (ONOO$^-$) (Junod, 1986; Powis, 1989). Unpaired electrons in the outer shell make ROS very unstable. They can react with other molecules to gain or lose electrons (Downey, 1990). ROS are capable of oxidizing proteins, lipids, and nucleic acids. Oxidation of proteins leads to changes in enzyme activity, and often leads to more rapid degradation of the respective protein (Stadtman, 1992). Lipid peroxidation damages cell membrane as well as extracellular lipids, and disrupts cellular homeostasis (Kappus, 1991). Oxidation of nucleic acids leads to strand breaks and hydroxylated nucleosides. Misrepair of the DNA lesion results in mutagenicity and even carcinogenesis (Halliwell, et.al, 1991). Therefore, the consequences of ROS exposure include altered gene regulation and signal transduction, apoptosis, necrosis, fibrosis, and carcinogenesis (Kaplowitz and Tsukamoto, 1996).
In most circumstances, ROS can be effectively scavenged by endogenous enzymatic and non-enzymatic antioxidants (Machlin and Bendich, 1987; Sies, 1997). However, in pathological situations, the generation of ROS overwhelms the antioxidant defense (Sies, 1997). The extent of tissue damage is the result of the imbalance between the free radicals generated and the antioxidant defense system (Machlin and Bendich, 1987; Stohs, 1995).
Defenses against ROS include enzymes, proteins, and radical scavengers. The enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase minimize exposure to ROS by converting $O_2^-$ and $H_2O_2$ to water (Halliwell, 1995). Auxiliary enzymes, including glutathione reductase, $\gamma$-glutamylcysteine synthetase, GSH synthetase, and glucose-6-phosphate dehydrogenase, contribute to the antioxidant defense through GSH and NADPH biosynthesis or regeneration (Kaplowitz, et.al., 1996). Tocopherol, ascorbic acid (reduced), beta-carotene, glutathione, bilirubin, and transferrin effectively scavenge ROS and terminate free radical reactions (Junod, 1986; Machlin and Bendich., 1987; Kaplowitz, et.al., 1996).

CYPs are an important generator of ROS (Sies, 1997). In addition to CYPs, there are several important sources for the generation of ROS. Electron outflow from mitochondria (Freeman and Crapo, 1982; Richter, 1995), metal accumulation (Kaplowitz and Tsukamoto, 1996), activation of nitric oxide synthetase (Bredt and Snyder, 1989), activation of arachidonic acid metabolism (Piomelli and Greengard, 1990), and activation of oxidases (Simonian and Coyle, 1996) all lead to the production of ROS.

The brain is more susceptible to oxidative damage than other organs. Brain tissue is characterized by high oxygen consumption, high content of
polyunsaturated lipids, accumulation of iron in certain brain regions, and relatively low catalase activity. All these features make the brain susceptible to oxidative injury (Halliwell, 1989). Oxidative stress occurring in neurodegenerative disorders shows remarkable cellular selectivity (Simonian and Coyle, 1996). Astrocytes maintain relatively high intracellular concentration of GSH and recycle ascorbate, which make astrocytes more resistant to oxidative damage than neurons and oligodendrocytes (Wilson, 1997; Peuchen, et.al., 1997). In addition, the neuroprotective effect of astrocytes may partially prevent neurons from oxidative damage (Wilson, 1997).

In contrast to the brain, the liver contains higher levels of the metabolic enzymes that generate ROS during mixed-function oxidation. However, the liver also has a much stronger antioxidative defense, including higher levels of glutathione and glutathione peroxidase (Makar, et.al., 1994).

**Glutamate and Glutamate Homeostasis:**

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). Glutamatergic transmission is involved in several important brain functions, such as learning and memory (Bliss and Collingridge, 1993). However, at elevated extracellular concentrations,
glutamate can produce neuronal damage, a phenomenon know as excitotoxicity (Choi, 1992). Several neurodegenerative diseases, including stroke, epilepsy, Huntington’s Disease, Amyotrophic Lateral Sclerosis (ALS), and Alzheimer’s Disease, are potentially linked to glutamatergic excitotoxicity (Gegelashvili and Schousboe, 1997).

Figure 3. Schematic model for glutamate regulation. NMDA, NMDA receptor; AMPA, AMPA receptor; KA, KA receptor; ACPD-G, G-protein coupled-AMPA receptor; PLC, phospholipase C. (Nicholls and Attwell, 1990)

Glutamate is distributed throughout the entire central nervous system (Monaghan, et.al., 1989). This amino acid neurotransmitter is packaged in synaptic vesicles within glutamatergic presynaptic terminals and released into
the synaptic cleft upon stimulation (Nicholls and Attwell, 1990). After release, glutamate activates receptors on pre- and postsynaptic neuronal and glial membranes. Excitatory transmission and complex signaling processes, such as long term potentiation (LTP), long term depression (LTD), and neuronal plasticity are evoked through the activation of glutamatergic ionotropic and metabotropic receptors (Cotman, et.al., 1989; Collingridge and Wolf, 1991). The amount of glutamate in the synaptic cleft is less than 1 \mu M in resting conditions, while the value can transiently reach 10^{-3} M when glutamate release is stimulated (Fonnum, 1984; Nicholls and Attwell, 1990). The glutamate in extracellular space is rapidly transported into astrocytes and presynaptic neurons by glutamate transporters located in neurons and glia. This action terminates glutamate synaptic transmission and prevents the extracellular glutamate concentration from reaching neurotoxic levels (Nicholls and Attwell, 1990). Glutamate is metabolized to glutamine by glutamine synthetase (GS), an astrocytic enzyme, via an ATP dependent mechanism. Glutamine is transported from astrocytes back to neurons and converted to glutamate by glutaminase in presynaptic neurons (Hamberger, et.al., 1979). The synthesis, release, uptake, and metabolism of glutamate constitute the glutamine cycle in CNS. Changes in the glutamine cycle, such
as increased release of glutamate, loss of the glutamate transporter, or inhibition of glutamine synthetase activity, may disrupt glutamate homeostasis in the CNS, leading to elevated extracellular glutamate and excitotoxicity (Bristol, and Rothstein, 1996; Trop, et.al., 1995). For example, inhibition of GS by methionine sulfoximine triggers convulsant activity (Rowe, et.al., 1969).

Growing evidence links oxidative stress and excitotoxic cell death (Dyken, et.al, 1987). Both GS and glutamate transporters have been reported to be inactivated by ROS in vivo and in vitro (Oliver, et.al., 1990; Sorg, 1997). Free radicals can also increase the release of glutamate, leading to increased glutamate in the synaptic cleft and prolonged glutamatergic signals (Volterra, et.al., 1994). Following the activation of kainic acid (KA) and N-methyl-D-aspartate (NMDA) receptors, the increase of intracellular \(\text{Ca}^{2+}\) leads to the activation of nitric oxide synthetase, xanthine oxidase, and release of arachidonic acid. These effects greatly increase the generation of ROS (Lafon-Cazal, et.al, 1993; Sun, et.al., 1992). Oxidative damage to lipids occurs following the activation of glutamate receptors (Puttfarcken, et.al., 1993). In addition, antioxidants protect neurons against excitotoxic cell death (Miyamoto, et.al., 1989)
Loss of GS activity is generally considered as a sensitive indicator of tissue oxidative injury (Oliver, et.al., 1990). The activity of GS, a key enzyme regulating glutamate homeostasis and ammonia metabolism in the CNS, decreases to 65% of control value after 2 hour reperfusion following 10 minutes ischemia. The reduction of GS activity is effectively prevented by preadministration of free radical spin trap reagents (Oliver, et.al., 1990). Oxidative inactivation of GS involves the oxidation of His-269, Arg-344, and unfolding of the peptide chain, which makes the enzyme susceptible to proteases (Roseman and Levine, 1987).

Proposed Research:

Increased CYP 1A1 activity is associated with elevated generation of ROS in 3-MC treated rats (Mishin, et.al., 1975), and induction of CYP 1A1 by TCDD in hepatoma cells has been reported to cause oxidative DNA damage (Park, et.al., 1996). All this evidence suggests that induction of CYP 1A1 may increase oxidative stress in vivo. The presence of CYP 1A1 in the CNS is well documented (Warner, et.al., 1991; Kohler, et.al, 1988). Investigation of the interaction between CYP induction and oxidative damage in the brain is a relatively new research area. Most research has been carried out on CYP 2E1, the enzyme induced by ethanol. However, the effect of ethanol on
oxidative damage is complicated by increased ROS formation by mitochondria (Kukielka, et al., 1994) as well as metabolism of ethanol by various oxidases (Montoliu, et al., 1995). The regulatory effect of CYP 1A1 induction on oxidative status of the brain has not been reported. In the present study, we investigated whether induction of CYP 1A1 can cause oxidative damage to lipids and proteins, and affect GS activity in the brain and liver. Although acute exposure to PAHs and HAHs does not lead to neurotoxicity (Izdebska-Szymona, et al, 1997; Unkila, et al., 1995), the investigation of their ability to cause or exacerbate oxidative damage may help to elucidate their role in other CNS pathologies.
Specific Objectives:

The overall goal of this project is to investigate the interaction between CYP 1A1 induction and oxidative damage in the brain and liver. We hypothesized that induction of CYP 1A1 following exposure to PAHs and HAHs causes oxidative damage, and alters GS activity in the brain.

The following questions were addressed in the project:

1. Does 3-MC induce CYP 1A1 in rat brain and liver? Does the dosing regimen affect the extent of CYP 1A1 induction in the brain and liver?

2. Does increased CYP 1A1 activity lead to more protein oxidation and lipid peroxidation in rat brain and liver?

3. Does increased CYP 1A1 activity change the susceptibility of the brain and liver to oxidative challenge?

4. Does induction of CYP 1A1 affect GS activity in rat brain and liver?
Materials and Methods

Materials:

All chemicals were obtained either from Sigma (St. Louis, MO) or from Fisher Scientific (Pittsburgh, PA) except 7-ethoxyresorufin (Biomol, Plymouth, PA) and protein assay dye reagent (Bio-Rad, Hercules, CA).

Animal Treatment:

Male Sprague-Dawley rats (150-200 g) were purchased from Simonsen Labs (Gilroy, CA). Rats were maintained on a 12 hour light-dark cycle with food and water ad libitum. Rats were allowed to adjust from shipping for 4-5 days prior to beginning of experiments. 3-MC was suspended in corn oil at a concentration of 55 µmol/ml. Corn oil or 3-MC was administered to rats using two different schemes:

1. Five daily intraperitoneal injections of 110 µmol/Kg 3-MC or 2 ml/kg corn oil (Dhawan, et.al., 1990).

2. A single intraperitoneal injection of 550 µmol/Kg 3-MC or 10 ml/Kg corn oil.

The rats were sacrificed one day, three days, five days, and ten days after the last injection. n = 6-12 for each treatment.
**Microsomal Protein Preparation:**

The rats were decapitated and the whole rat brain and tissues from the biggest lobe of liver were quickly removed and rinsed in homogenization buffer (150 mM NaCl, 67 mM Na$_2$HPO$_4$, 1 mM EDTA, 0.1 mM PMSF and 20% glycerol, pH = 7.4). One hemisphere of the brain and half of the liver tissue were quickly frozen in a dry ice-ethanol bath and stored at -80 °C. The other half of the tissues were used for brain and liver microsomal protein preparation.

Rat brain and liver microsomes were prepared as described by Zhang (1997). The brain and liver tissues were minced in approximately 15 ml of homogenization buffer and homogenized by 10-12 strokes using a 15 ml Wheateon Glass/Glass homogenizer and a Teflon pestle. Tissue homogenates were centrifuged at 2000 x g for 10 minutes to remove cell debris, followed by centrifugation of the supernatants at 10,000 x g for 20 minutes to remove mitochondria and nuclei. The supernatants were centrifuged at 110,000 x g in an ultra-centrifuge for 60 minutes. All centrifugations were carried out at 4 °C. The resulting supernatants were saved and stored at -80 °C for glutamine synthetase activity assay. The pellets were suspended in 800 μl of microsomal suspension buffer (67 mM...
$\text{Na}_2\text{HPO}_4$, 1 mM EDTA, 0.1 mM pepstatin, 0.1 mM leupeptin, 0.1 mM PMSF and 20% glycerol, pH = 7.4) and transferred to a 1 ml glass homogenizer. After 6-7 strokes for homogenization, the microsomal protein suspensions were stored at -80 °C for no more than 30 days before assaying for CYP 1A1 activity.

**CYP 1A1 Activity Assay:**

The CYP 1A1 activity in rat brain and liver microsomal proteins were quantified by the ethoxyresorufin-O-deethylase (EROD) method as described by Unkila with minor changes (Unkila, et.al., 1993). Aliquots (0.2 mg protein for brain microsomes and 0.04 mg protein for liver microsomes) of the microsomal suspensions in 67 mM sodium phosphate buffer (total volume 1 ml) were kept on ice. After the addition of 10 µl of 0.5 µM ethoxyresorufin ethanol solution, the mixtures were transferred to a semimicro polystyrene cuvette and incubated for 10 minutes at 37 °C. The assay was initiated by adding 20 µl 50 mM NADPH. The reaction mixtures were incubated at 37 °C for 15 minutes for brain microsomes and 10 minutes for liver microsomes. Resulting fluorescence was measured at 585 nm with excitation at 550 nm using an Hitach F2000 fluorescence spectrophotometer. Non-specific
fluorescence for the samples was measured using boiled, denatured samples following the same protocol.

The amount of resorufin produced in the tissue homogenate suspensions was calculated based on a standard curve developed from a range of resorufin concentration in the presence of 5 μM ethoxyresorufin and 1 mM NADPH. Standards ranged from 0-15 nM for brain microsomes, 0-3 μM for 3-MC treated rat liver microsomes, and 0-50 nM resorufin for corn oil treated rat liver microsomes.

To confirm the assay condition, EROD assay was also carried out after incubating some brain or liver microsomes at 37 °C with 50 μM α-NF, a potent CYP 1A1 inhibitor, for ten minutes.

The time-course relationship for CYP 1A1 activity in rat brain and liver was established for both schemes. The 3-MC-treated rats with maximum CYP 1A1 activity in the brain or liver and the corresponding corn oil-treated controls were further used for lipid peroxidation and protein oxidation assays.

**Lipid Peroxidation Assay:**

The level of lipid peroxidation in rat brain and liver was quantified by the thiobarbituric acid method (Buege, et.al., 1978). Frozen rat brain and liver tissues prepared as previously described were thawed at room
liver tissues prepared as previously described were thawed at room temperature. The tissues were cut into small pieces and rinsed twice with 5 ml 67 mM sodium phosphate buffer (pH = 7.4). The tissues were homogenized for 8-10 stokes in about 5 ml homogenization buffer at 4 °C. Aliquots (1.5 mg protein) of tissue homogenates were mixed with 0.05 M Tris-HCl buffer (pH = 7.4) to a final volume of 1.5 ml. The reaction were allowed to proceed at 37 °C for 10 minutes. Aliquots (1.5 ml) of TCA-TBA-HCl reagent (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid and 0.25 M HCl) were then added into test tubes on ice. The reaction mixtures were vortexed vigorously and moved to an 80 °C water bath for 15 minutes. After stopping the reaction on ice, the reaction mixtures were centrifuged at 3000 rpm for 5 minutes to remove the flocculent precipitate. The absorbance of the supernatant was measured at 535 nm using a Beckman DU650 spectrophotometer. Absorbance was corrected by a blank, which contained all the reagents except the tissue homogenates.

The amount of thiobarbituric acid-reactive substance (TBARS) in tissue homogenates was determined by comparison with a standard curve of a range of MDA concentrations from 0-5 nM. MDA standard was prepared by hydrolyzing malonaldehyde bis [dimethyl acetal] in 0.05 M Tris-HCl buffer.
Protein Oxidation Assay:

Protein oxidation in rat brain and liver was quantified by the loss of tryptophan fluorescence as described by Davies (Davies and Lin, 1987). Aliquots of tissue homogenates (0.7 mg protein) in 67 mM sodium phosphate buffer (total volume 1.5 ml) were mixed with 100 μl 20% sodium dodecyl sulfate (SDS) and incubated at 37 °C for 30 minutes. Fluorescence for tryptophan in the samples was measured at 340 nm with excitation at 280 nm using a Hitachi F2000 fluorescence spectrophotometer.

The effect of 3-MC treatment on protein oxidation was determined by comparing tryptophan fluorescence between corn oil-treated and 3-MC-treated rats.

Oxidative Challenge:

The brain and liver homogenates (1.5 mg protein), prepared as described in the lipid peroxidation assay, were incubated at 37 °C for 10 minutes. Ten microliters of 15 mM NADPH, 10 μl of 15 mM FeCl₃, and 50 μl of 51 mM ADP were added to tissue homogenates and incubated at 37 °C for 10 more minutes. The amounts of MDA generated were examined by reacting with thiobarbituric acid as described in the lipid peroxidation assay. In order to examine the effect of CYP 1A1 on lipid peroxidation under
oxidative challenge, α-NF (50 μM) was incubated with tissue homogenates for ten minutes before oxidative challenge.

Protein oxidation was also investigated by measuring tryptophan fluorescence after oxidative challenge. Brain and liver homogenates (0.7 mg protein) in 67 mM sodium phosphate buffer (total volume 1.5 ml) were first incubated with 100 μl of 20% sodium dodecyl sulfate (SDS) at 37 °C for 20 minutes. Then 10 μl of 15 mM NADPH, 10 μl of 15 mM FeCl₃, and 50 μl of 51 mM ADP were added to tissue homogenates and incubated at 37 °C for 10 more minutes. Tryptophan fluorescence was measured as previously described.

Glutamine Synthetase Assay:

Glutamine synthetase activity was determined by the method of Meister (1985). The supernatants from the 110,000 x g centrifugation in the microsomal preparation were used to determine GS activity. Aliquots (0.4 mg protein) of the supernatants were added to 750 μl of a solution containing 0.1 M L-glutamine, 0.125 M hydroxylamine, 0.4 mM manganese chloride, 20 mM arsenic acid and 0.8 mM ADP. Sufficient volume of 67 mM sodium phosphate buffer (pH=7.4) was added to make the final volume 1.5 ml. The reaction was carried out at 37 °C for 60 minutes. The reaction was stopped
by removing the samples to an ice bath and adding 1.5 ml of 0.37 M ferric chloride. After allowing 30 minutes for color development, samples were centrifuged at 3000 rpm for 5 minutes to remove any debris. The absorbance at 555 nm for the supernatants was read on a Beckman DU 650 spectrophotometer.

The amount of γ-glutamyl hydroxymate (g-GH) produced was determined by comparison with a standard curve of a range of g-GH concentrations from 0-0.8 μM.

**Protein Assay:**

The protein content in samples were determined by the Bradford method (Bradford, 1976). Samples and protein standards were reacted with four-fold diluted protein assay dye reagent for 10 minutes at room temperature. The absorbance for the reaction mixtures was measured at 595 nm by a Beckman DU650 spectrophotometer.

**Statistical Analysis:**

Statistical analysis was carried out using the GBSTAT ® program. Treatment means were compared using ANOVA and the Newman-Keuls test.
Results:

Identification of CYP 1A1 Reaction Conditions:

Preliminary studies were performed to optimize the EROD assay conditions. Table 1 shows that the highest CYP 1A1 activity after 10 minutes of incubation was observed with 0.2 mg of brain and 0.04 mg of liver microsomal protein. Using these established amounts of protein, the reaction was allowed to proceed 2-30 minutes. Maximum CYP 1A1 activity occurred at 15 minutes in brain microsomes and 10 minutes in liver microsomes (Table 2). Therefore, 0.2 mg protein and 15 minutes reaction time were used in EROD assays for brain microsomes, and 0.04 mg protein and 10 minutes reaction time with liver microsomes.

Multiple Injection Protocol:

The induction of CYP 1A1 in rat brain and liver by 5 daily injections of 3-MC are shown in Figure 4 and Figure 5. Three days after the last dose, 3-MC induced approximately a 7-fold increase in brain CYP 1A1 activity. CYP 1A1 activity was 1.092 ± 0.155 pmol resorufin/min/mg protein following 3-MC treatment and 0.173 ± 0.06 pmol resorufin/min/mg protein in corn oil controls. No significant CYP 1A1 induction occurred one day, five days, or ten days after multiple doses of 3-MC in rat brain. In contrast, CYP
1A1 activity was increased in 3-MC treated rat liver one day after the treatment. 3-MC treatment increased CYP 1A1 activity about 60-100 fold at all time points. The increased enzyme activity in the brain and liver was effectively inhibited by 50 μM α-NF, a selective CYP 1A1 inhibitor (Table 3).

Figures 6 and 7 show the amount of lipid peroxidation in rat brain and liver following 3-MC treatment, and the additional thiobarbituric acid-reactive substances (TBARS) generation following oxidative challenge. Three days after dosing, the time when maximum CYP 1A1 activity was observed in both brain and liver, 3-MC treatment did not change the amount of lipid peroxidation in either tissue. The rate of TBARS generation was 0.27-0.3 nmol/min/mg protein in the brain and 0.16-0.21 nmol/min/mg protein in the liver. 3-MC treatment did not increase lipid peroxidation over that seen with corn oil-treated rats. In rat brain, oxidative challenge significantly increased the levels of lipid peroxidation in 3-MC and corn oil-treated rats. However, this increase was not inhibited by 50 μM α-NF in either case. In rat liver, the level of lipid peroxidation did not increase following oxidative challenge and the addition of 50 μM α-NF did not change the rate of TBARS generation after oxidative challenge.
Figure 8 shows the extent of protein oxidation in rat brain and liver 3 days after 3-MC and corn oil treatment. The loss of tryptophan fluorescence in tissue homogenates serves as a marker for protein oxidation. 3-MC did not increase brain protein oxidation. 3-MC treatment produced a 7.7% decrease in tryptophan fluorescence in rat liver protein. The tryptophan fluorescence is 115 ± 0.787 in 3-MC-treated rats, which is significantly lower than the 125 ± 2.62 value of corn oil-treated rats.

Figures 9 and 10 show the effect of 3-MC treatment on GS activity in the brain and liver. Neither brain nor liver GS activity was changed following the induction of CYP 1A1 by multiple injections of 3-MC. Average GS activity was 28-40 nmol/min/mg protein in rat brain, and 6-9 nmol/min/mg protein in rat liver in both 3-MC and corn oil groups.

**Single Dose Protocol:**

A single dose of 3-MC did not increase CYP 1A1 activity in rat brain at any measured time point. The brain CYP 1A1 activity was almost unmeasurable in most rats. Conversely, 3-MC caused a marked induction of CYP 1A1 in rat liver. A 55-75 fold increase in CYP 1A1 activity was observed one day, three days, five days, and ten days after treatment (Fig. 30).
11). CYP 1A1 activity was relatively close to the corresponding values in the multiple injection protocol.

Figure 12 and figure 13 present data showing that a single injection of 3-MC did not increase lipid peroxidation in rat brain and liver. A single dose of 3-MC produced similar levels and a similar pattern of lipid peroxidation as that seen with rats receiving multiple doses of 3-MC.

The effect of a single dose of 3-MC on protein oxidation was investigated in the brain and liver (Fig. 14). 3-MC exposure did not increase protein oxidation in rat brain five days after treatment, while significantly decrease liver tryptophan fluorescence to 91.6% of the value seen with corn oil-treated rats.

Oxidative challenge significantly oxidized brain protein (Fig. 15). The tryptophan fluorescence markedly decreased from 166.8 ± 1.84 to 28.3 ± 1.24 following exposure to ADP, iron, and NADPH.

GS activity in rat brain and liver was not altered by 3-MC treatment at any time point (Fig. 16, Fig. 17). The values of 23-30 nmol/min/mg protein and 6-7 nmol/min/mg protein for the brain and liver GS activity respectively, were in the same range as the values from the first treatment protocol.
Table 1. Effect of Protein Content on CYP 1A1 Activity:

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Protein (mg)</th>
<th>Reaction Time (min)</th>
<th>CYP 1A1 Activity (pmol resorufin/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.2</td>
<td>10</td>
<td>1.32 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>10</td>
<td>** 0.83 ± 0.16</td>
</tr>
<tr>
<td>Liver</td>
<td>0.04</td>
<td>10</td>
<td>4.73 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>10</td>
<td>3.87 ± 0.38</td>
</tr>
</tbody>
</table>

** = Significantly different from the CYP 1A1 activity measured at 0.2 mg protein (P < 0.05).
Table 2. Effect of Reaction Time on CYP 1A1 Activity.

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Protein (mg)</th>
<th>Reaction Time (min)</th>
<th>CYP 1A1 Activity (pmol resorufin/min/mg protein)</th>
<th>CYP 1A1 Activity (nmol resorufin/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.2</td>
<td>2</td>
<td>** 0</td>
<td>** 2.40 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>5</td>
<td>* 0.98 ± 0.26</td>
<td>3.72 ± 0.83</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>10</td>
<td>1.13 ± 0.30</td>
<td>4.72 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>15</td>
<td>1.32 ± 0.22</td>
<td>4.20 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>30</td>
<td>* 0.96 ± 0.11</td>
<td>* 3.06 ± 0.15</td>
</tr>
<tr>
<td>Liver</td>
<td>0.04</td>
<td>2</td>
<td>** 0.98 ± 0.26</td>
<td>** 3.72 ± 0.83</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>5</td>
<td>* 1.13 ± 0.30</td>
<td>4.72 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>10</td>
<td>1.32 ± 0.22</td>
<td>4.20 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>15</td>
<td>* 0.96 ± 0.11</td>
<td>* 3.06 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = CYP 1A1 activity is significantly different from the highest value (* P < 0.05, ** P < 0.01)
Figure 4. Effect of 5 daily injections of 3-MC on CYP 1A1 activity in rat brain. CYP 1A1 activity in rat brain was determined 1 day, 3 days, 5 days, and 10 days after 3-MC treatment. CYP 1A1 activity was expressed as pmol resorufin generated/min/mg protein. Each data point represents mean ± SEM (n = 6-12). ** = CYP 1A1 activity significantly increased over corresponding values from corn oil-treated rats (P < 0.01).
**Figure 5.** Effect of 5 daily injections of 3-MC on CYP1A1 activity in rat liver. CYP 1A1 activity in rat liver was determined 1 day, 3 days, 5 days, and 10 days after 3-MC treatment. CYP 1A1 activity was expressed as nmol resorufin generated/min/mg protein. Each data point represents mean ± SEM (n = 6-12). ** = CYP 1A1 activity significantly increased over corresponding values from corn oil-treated rats. # # = CYP 1A1 activity is significantly higher than any other time point (P < 0.01).
Table 3. Effect of α-NF on CYP 1A1 Activity.

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Protein (mg)</th>
<th>α-NF (µM)</th>
<th>CYP 1A1 Activity (nmol resorufin/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.2</td>
<td>0</td>
<td>1.09 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>50</td>
<td>** 0</td>
</tr>
<tr>
<td>Liver</td>
<td>0.04</td>
<td>0</td>
<td>4.70 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>50</td>
<td>** 0.054 ± 0.003</td>
</tr>
</tbody>
</table>

** = The CYP 1A1 activity is significantly different than that seen without α-NF (P < 0.01).
Figure 6. Lipid peroxidation in 3-MC and corn oil treated rat brain. The amount of lipid peroxidation in 3-MC and corn oil treated rats brain was evaluated 3 days after multiple injections, and expressed as nmol TBARS/min/mg protein. CONT = extent of lipid peroxidation without oxidative challenge. OX = lipid peroxidation following oxidative challenge. OX + αNF = lipid peroxidation in the brain after treating with oxidative challenge and α-NF. Each data point represents mean ± SEM (n=6-7). ** = The amount of lipid peroxidation significantly increased over corresponding CONT value (P < 0.01).
Figure 7. Lipid peroxidation in 3-MC and corn oil treated rat liver. Lipid peroxidation was examined in 3-MC and corn oil treated rat liver 3 days after multiple injections. The amount of lipid peroxidation was shown as nmol TBARS/min/mg protein. Assay conditions were the same as in Fig. 6. Each data point represents mean ± SEM (n=6-7).
Figure 8. Protein oxidation in 3-MC and corn oil-treated rat brain and liver. Protein oxidation was examined in the brain and liver 3 days after treatment. The amount of protein oxidation were determined by the loss of tryptophan fluorescence at 345 nm. Each data point represents mean ± SEM (n=6-7). * = The level of tryptophan fluorescence significantly different from corn oil group (* P < 0.05).
Figure 9. GS activity in 3-MC and corn oil-treated rat brain. GS activity in rat brain was determined 1 day, 3 days, 5 days, and 10 days after 5 doses of 3-MC or corn oil. GS activity is expressed as nmol γ-glutamyl hydroxymate (g-GH) generated /min/mg protein. Each data point represents mean ± SEM (n=6-7).
Figure 10. GS activity in 3-MC and corn oil treated rat liver. GS activity in rat liver was determined 1 day, 3 days, 5 days, and 10 days after 5 doses of 3-MC or corn oil. GS activity is expressed as nmol γ-glutamyl hydroxymate (g-GH) generated/min/mg protein. Each data point represents mean ± SEM (n=6-7).
Figure 11. Effect of a single dose of 3-MC on CYP 1A1 activity in rat liver. CYP 1A1 activity in rat liver was determined 1 day, 3 days, 5 days, and 10 days after the injection. CYP 1A1 activity was expressed as nmol resorufin generated/min/mg protein. Each data point represents mean ± SEM (n = 6). ** = CYP 1A1 activity significantly increased over corresponding values from corn oil-treated rats (P < 0.01).
Figure 12. Lipid peroxidation in 3-MC and corn oil treated rat brain. Lipid peroxidation was evaluated in rat brain 5 days after the single injection. The amount of lipid peroxidation was expressed as nmol TBARS/min/mg protein. CONT = extent of lipid peroxidation without oxidative challenge. OX = lipid peroxidation following oxidative challenge. OX + αNF = lipid peroxidation in the brain after treating with oxidative challenge and α-NF. Each data point represents mean ± SEM (n=6). ** = The amount of lipid peroxidation significantly increased over corresponding CONT value (P < 0.01).
Figure 13. Lipid peroxidation in 3-MC and corn oil treated rat liver. Lipid peroxidation was examined in rat liver 5 days after the single injection. The amount of lipid peroxidation was expressed as nmol TBARS/min/mg protein. Assay condition were same as in Fig. 12. Each data point represents mean ± SEM (n=6).
Figure 14. Protein oxidation in 3-MC and corn oil treated rat liver. Protein oxidation was examined in the brain and liver 5 days after a single injection of 3-MC. The amount of protein oxidation were determined by loss of tryptophan fluorescence at 345 nm. Each data point represents mean ± SEM (n=6). ** = The level of tryptophan fluorescence significantly different from corn oil (P < 0.01).
Figure 15. Effect of oxidative challenge on protein oxidation. The extent of protein oxidation in rat brain was examined following oxidative challenge. Each data point represents mean ± SEM (n=4-6). ** = Protein oxidation was significantly increased by oxidative challenge (P < 0.01).
Figure 16. GS activity in 3-MC and corn oil treated rat brain. GS activity in rat brain was determined 1 day, 3 days, 5 days, and 10 days after a single dose of 3-MC or corn oil. GS activity is expressed as nmol γ-glutamyl hydroxymate (g-GH) generated/min/mg protein. Each data point represents mean ± SEM (n=6).
Figure 17. GS activity in 3-MC and corn oil treated rat liver. GS activity in rat liver was determined 1 day, 3 days, 5 days, and 10 days after a single dose of 3-MC or corn oil. GS activity is expressed as nmol γ-glutamyl hydroxamate (g-GH) generated/min/mg protein. Each data point represents mean ± SEM (n=6).
Discussion

Two well established protocols were used to induce CYP 1A1 in the brain and liver. Both protocols exposed rats to a total of 550 μmol/kg of 3-MC. Although this dose is much larger than the estimated environmental exposure to PAHs and HAHs (nmol/Kg - pmol/Kg) (McFarland and Clarke, 1989), it is in the same range as the doses that were used in other studies to produce significant increases in CYP 1A1 activity in the rat brain and liver (Dhawan, et.al., 1990; Suarez, et.al., 1975).

CYP 1A1 activity in the brain and liver was examined one day, three days, five days, and ten days after 3-MC treatment (see Figs. 4, 5, and 11). In order to test whether the dosing regimen affected CYP 1A1 induction and the extent of oxidative damage in tissues, 3-MC (550 μmol/Kg) was administered to rats either by five daily injections or a single injection. Induction of CYP 1A1 in the brain and liver had different time-course relationships after these two injection protocols. A significant increase in CYP 1A1 activity in rat brain was detected three days after five daily injections of 3-MC, while brain CYP 1A1 activity did not increase at all time points after a single dose of 3-MC. Three days after 5 daily injections of 3-MC, rat brain CYP 1A1 activity was 1.092 ± 0.155 pmol/min/mg protein in
our study, which is similar to 1.44 ± 0.5 pmol/min/mg protein reported by Perrin, et.al. (1990). Dramatically increased CYP 1A1 activity was observed in rat liver at all time points after both protocols. The extent of hepatic enzyme induction produced by the single injection protocol was less than that seen three days after the multiple injection protocol.

The responses of the brain and liver to 3-MC treatment were remarkably different. A relatively transient increase in CYP 1A1 activity in the brain was in contrast to much more persistent induction of CYP 1A1 in the liver. 3-MC treatment increased CYP 1A1 activity seven fold in the rat brain, which is much lower than the 50-100 fold increase of CYP 1A1 activity in the liver. At all time points after both injection protocols, CYP 1A1 activity ranged from 0-0.173 pmol/min/mg protein and 30-45 pmol/min/mg protein in the control rat brain and liver, respectively. These data are slightly lower than 0.45-0.55 pmol/min/mg protein and 60 pmol/min/mg protein reported by Perrin, et.al. (1990), using a slightly different injection protocol (0.5 ml corn oil for 3 days).

Neither treatment protocol led to a sustained increase in CYP 1A1 activity in the brain. One possible explanation for the short duration of CYP induction in the brain is that CYP 1A1 can be destroyed during lipid
peroxidation (Bast and Haenen, 1984). Another possible explanation is that the metabolism of 3-MC may be accelerated by increased CYP 1A1 activity. Although we did not observe increased CYP 1A1 activity in the brain after a single dose of 3-MC, it is still possible that the induction of CYP 1A1 occurred at a time that was not chosen in our protocol. Also, when comparing the results of the two protocols in the liver, the multiple dose regimen induced more CYP 1A1 activity. Thus it is possible that the single dose protocol was not sufficiently rigorous to induce CYP 1A1 in the brain.

Although the brain is particularly vulnerable to lipid peroxidation, TBARS generation did not change following increased CYP 1A1 activity (see Fig. 6). Our finding implies that endogenous antioxidant defense can protect the brain from ROS attack of the magnitude that can be generated by induction of CYP 1A1. Another possible reason for the lack of increase in lipid peroxidation is that 3-MC treatment may enhance antioxidant defenses by changing the levels of antioxidants. For example, 3-MC transcriptionally induces glutathione S-transferase (GST) via AhR (Okey, et.al., 1994). Increased GST enhances the secretion of 3-MC metabolites, inhibits lipid peroxidation by favoring the reduction of lipid hydroperoxides to stable lipid.
aldehydes, and protects against lipid peroxidation by enhancing the secretion of cytotoxic aldehyde products (Nordmann, 1994).

Induction of another CYP isoform is linked with oxidative challenges in rat brain. Montoliu, et.al. (1994) demonstrated that rats exposed to ethanol for 14 days exhibited induction of CYP 2E1, increased ROS formation, and depletion of GSH in the brain. The increased formation of ROS after ethanol treatment can be partially inhibited in vitro by CYP 2E1 antibody. However, chronic alcohol consumption did not enhance the production of TBARS. Conversely, later studies by Montoliu, et.al. (1995) demonstrated that fourteen day exposure to 25 mM or 50 mM ethanol induced CYP 2E1 and increased lipid peroxidation in astrocytes. However, this study did not address the role of oxidases other than CYP 2E1 in ethanol metabolism.

In spite of large increases in CYP 1A1 activity, neither 5 daily injections nor a single dose of 3-MC caused measurable lipid peroxidation in the rat liver (see Figs. 7, 13). In our studies, the level of TBARS in the corn oil-treated rat liver is similar to the value 0.02-0.04 nmol/min/mg protein detected by Iba (1988) in rat liver three days after receiving three daily injections of 0.4 ml of corn oil. Our result is consistent with previous studies showing that treatment with 150 μmol/Kg 3-MC for two days did not produce
lipid peroxidation in rat liver (Suarez, et.al., 1975; Junqueira, et.al., 1991). Both results suggest that the hepatic antioxidant defense can effectively scavenge oxygen radicals generated by increased CYP 1A1. Induction of glutathione S-transferase (GST) by 3-MC in the liver may also play a role in preventing oxidative damage in the liver.

The minor involvement of CYP 1A1 in lipid peroxidation was confirmed by assessing its ability to promote lipid peroxidation following oxidative challenge (see Figs. 6, 7, 13). The extent of lipid peroxidation was examined after challenging the brain and liver homogenates with ferric chloride, ADP, and NADPH, an oxidative challenge system shown by Svingen to significantly increase CYP-mediated lipid peroxidation in microsomes (Svingen, et.al., 1979). In this oxidative challenge system, NADPH promotes CYP 1A1 activity, ferric chloride catalyzes the generation of hydroxyl radicals from H$_2$O$_2$, and facilitates the decomposition of lipid hydroperoxides to peroxyl and alkoxy radicals (Reiter, 1995). ADP enhances the electron outflow from mitochondria (Simonian and Coyle, 1996). In our study, oxidative challenge dramatically increased the extent of lipid peroxidation in the brain, while no change was seen in the liver. CYP 1A1 induction by 3-MC did not lead to more lipid peroxidation in either the
brain or liver. The effect of oxidative challenge on lipid peroxidation could not be inhibited by 50 μM α-NF, a potent CYP 1A1 inhibitor. These results indicate that induction of CYP 1A1 following 3-MC treatment did not exacerbate lipid peroxidation in the brain and liver under oxidative stress, implying that CYP 1A1 induction can not alter the outcome of oxidative stress associated with CNS or hepatic diseases.

Protein oxidation was measured by tryptophan fluorescence method of Teale (1959). Tryptophan fluorescence accounts for about 95% of the total fluorescence at 345 nm induced by excitation at 280 nm (Teale, 1959). Neither 3-MC injection protocol increased brain protein oxidation in our study (see Figs. 8, 14). Increased CYP 1A1 activity in the brain following 3-MC treatment did not change protein tryptophan fluorescence, suggesting that, similar to lipids, the induction of CYP 1A1 does not produce measurable brain protein oxidation.

In rat liver, both 3-MC-injection protocols decreased the protein tryptophan fluorescence by 8-9% (see Fig. 8, 14). This small reduction was in contrast to an 80% decrease in tryptophan fluorescence following oxidative challenge (see Fig. 15). Thus the 8-9% reduction in tryptophan fluorescence dose not represent major oxidation of hepatic protein.
Additional evidence that CYP 1A1 induction did not cause significant protein oxidation was found by assessing GS activity. The enzyme activity was not changed by either injection protocol in the rat brain and liver (see Figs. 9, 10, 16, 17). Brain GS activity ranged from 25-40 nmol/min/mg protein and liver GS from 6-9 nmol/min/mg protein in 3-MC and corn oil-treated rats. These values are consistent with data from others studies. e.g., 22-35 nmol/min/mg protein for brain GS, and 7-22 nmol/min/mg protein for liver GS (Wong, et.al., 1980; Wong and Dunn, 1977). GS is very susceptible to oxidative damage (Oliver, et.al., 1990) and oxidized GS is prone to cleavage by proteases (Lee, et.al., 1988). However, in our study, elevated CYP 1A1 activity in rat brain observed three days after multiple doses of 3-MC did not affect brain GS. This result is in accordance with the previous study by Bondy and Guo (1994). They reported that intraperitoneal injection of 4.5 g/Kg ethanol did not change brain GS activity 2 hours and 18 hours after treatment. Under the same conditions, ROS formation was increased and GS activity was decreased in the liver.

GS is located in astrocytes in CNS, an environment with relatively high antioxidant defenses. This may be an explanation for the stable GS activity in the brain following 3-MC treatment. Astrocytes accumulate α-tocopherol.

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and ascorbate, contain more glutathione, and exhibit higher GST activity than neurons (Wilson, 1997). If more ROS are generated by increased CYP 1A1 activity, the greater antioxidant defense in astrocytes may prevent detectable damage. In addition, the amount of ROS generated in astrocytes has not been quantified. Although the minor induction of CYP in the brain may lead to more ROS formation, we are unable to compare the increased generation of ROS from CYP 1A1 with more severe oxidative injury models that decrease GS activity by 40% (Oliver, et.al., 1990).

The rat liver showed extensive CYP 1A1 induction after 3-MC treatment, which may significantly increase the formation of ROS. However, neither multiple doses nor a single dose of 3-MC changed hepatic GS activity in our study (see Figs. 10, 17). The levels of GSH, GSH-metabolizing enzymes, and catalase are higher in the liver than in the brain (Kretzsgnlar, 1996). ROS generated by CYP 1A1 may be effectively scavenged by these antioxidants. Studies by Bondy and Guo (1994) showed that acute ethanol or acetaldehyde treatment cause significant depression of GS in the rat liver. Possible reasons for the difference seen with CYP 1A1 and CYP 2E1 induction include the generation of ROS during ethanol or acetaldehyde
metabolism by various oxidases, as well as increased ROS formation from mitochondria (Montoliu, et.al, 1995; Kukielka, et.al., 1994).

The lack of an effect of CYP 1A1 induction on brain GS activity indicates that exposure to 3-MC does not alter this important component in glutamate homeostasis. Moreover, the induction of a much higher level of CYP 1A1 activity also failed to affect the much lower level of GS activity found in liver. Although GS represents only one part of the glutamine cycle, the lack of evidence that CYP 1A1 produces any oxidative damage makes it unlikely that CYP 1A1 induction has any significant effect on glutamate homeostasis. These observations also make it unlikely that environmental exposure to inducers of CYP 1A1 can influence the excitotoxic component of neurologic disorders.

In conclusion, this project explored potential links between PAH exposure, oxidative damage, and glutamate homeostasis. Our studies demonstrated that induction of CYP 1A1 following 3-MC treatment did not oxidize either proteins or lipids, or affect GS activity in the rat brain and liver. The dosing regimen of 3-MC affected the induction of CYP 1A1 in the brain and liver, while no effect on the extent of oxidative damage was seen in either tissue. Furthermore, increased CYP 1A1 activity did not change the extent of
lipid peroxidation in the brain and liver following oxidative challenge. The inability of 3-MC to depress brain GS suggests that induction of CYP 1A1 does not disrupt glutamate homeostasis through this important component. If increased CYP 1A1 activity in the brain and liver following 3-MC treatment leads to increased ROS formation, the increase is insufficient to overwhelm the endogenous antioxidant defense system, produce detectable oxidative damage, and alter glutamate homeostasis.
Bibliography


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