Genomic analysis of human neuroblastoma cells in response to paraoxon exposure

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GENOMIC ANALYSIS OF HUMAN NEUROBLASTOMA CELLS IN RESPONSE TO PARAOXON EXPOSURE

by

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B.A. University of Montana, 2001

presented in partial fulfillment of the requirements

for the degree of

Master of Science

The University of Montana

July 2006

Approved by:

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8-23-06

Date
Genomic analysis of human neuroblastoma cells in response to paraoxon exposure

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Organophosphates (OP) belong to a diverse class of chemical compounds with applications for pesticide use as well as nerve gas agents. OPs are well-characterized neurotoxicants whose primary molecular target is acetylcholinesterase (AChE). By covalent modification of the enzymatic active site, OPs inhibit the hydrolytic function of AChE, resulting in acute toxic symptoms of hypercholinergic stimulation. We hypothesize that OPs are highly reactive compounds capable of interacting with other cellular proteins and eliciting additional, non-AChE-associated effects in neuronal cells. To begin to address this question, a human neuroblastoma cell line, SH-SY5Y, was exposed to 10 nM paraoxon (PX) for 3, 8, and 24 hours and changes in gene expression were analyzed using the Human Tox cDNA microarray chip. From the microarray data, a select group of genes, including ECM1 and ODC1, that showed differential expression following PX exposure were analyzed by semi-quantitative polymerase chain reaction and immunoblot. Although microarray data was not confirmed with RT-PCR, ECM1 showed increased protein expression following PX exposure. A comprehensive analysis of the microarray data using GoMiner software identified a number of possible cellular processes targeted by PX. One of these processes, cellular proliferation, was further characterized and it was demonstrated that 10 nM PX significantly induces proliferation in SH-SY5Y cells.
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ACKNOWLEDGMENTS

I would like to gratefully acknowledge the help and support received from a number of people, which, without their efforts this research project would not have been possible. First and foremost is my extraordinary research advisor, Dr. Katie George. The combination of her exceptional guidance, support and most of all friendship has made my life as a graduate student tremendously enjoyable. I would like to thank her for all she has done for me as both a student and friend. I would also like to generously thank my lab mate / partner in crime / exceptional friend, Sarj Patel. His presence and camaraderie in the lab made each day of work something to look forward to. His insightful advice and immeasurable knowledge were imperative to the completion of this project. Special thanks to Corbin Schwanke for helping me understand the ways and wonders of microarray technology. His microarray prowess was essential to this project. I express my gratitude to my committee members, Dr. Mark Pershouse and Dr. Scott Samuels, for their time, astute advice, and discussions. Thanks to Dr. Vernon Grund and the Department of Biomedical and Pharmaceutical Sciences for the opportunity to study and work in such a great school and location. To all my fellow graduate students, I express my genuine appreciation of your support and friendship. A special mention for Dr. Charles Thompson (and the Thompson Group), who gave me the initial opportunity to perform research and get excited about the possibility of becoming a science graduate student. Thanks to Jason Mullins (aka Arnold) for waking up at 6:30 every morning of the week to go putz around the gym and try to get huge with me. Inimitable gratefulness to my wonderful family, Joan, Bud, Kevin, and Jamie, who have instilled the spirit in me
to succeed while also being sure to enjoy life to the fullest. And finally, I express my
infinite gratitude to my beautiful and caring wife Thaedra. Her endless patience, love and
support during my time in graduate school will forever be remembered.
Chapter 1

INTRODUCTION

The development of organic phosphorus (organophosphates, OP) compounds originated during the middle of the 19th century with Jean Louis Lassaigne's analysis of the reaction of alcohol with phosphoric acid. The possible use of OP's as insecticides or nerve agents was realized in 1936 through the work of German chemist Gerhard Schrader (Rusyniak and Nanagas 2004). Schrader was in charge of developing new and improved insecticides when he created the compound Ethyl N,N-dimethylphosphoramidocyanidate (tabun) 1 (Figure 1.1), which became better known as nerve agent GA. Because of the time period, much of the continued effort in developing OPs was steered towards compounds that could be used as chemical warfare agents, although fully understanding the potential of OPs as insecticides. The potential of OPs to be used as chemical agents led to the German Ministry of Defense classifying Schrader’s OP compounds as confidential which initially prevented their development as commercial insecticides. Further work by Schrader resulted in the additional nerve agents 2-(fluoro-methyl-phosphoryl)oxypropane (sarin, GB) 2 and 3-(fluoro-methyl-phosphoryl)oxy-2,2-dimethyl-butane (soman, GD) 3 (Talley 2001).
Many of these OP compounds have been stockpiled for years as neurotoxic agents or nerve gas for military use (Caglioti 1983). Despite an international ban on the use and production of these neurotoxic compounds, there is evidence that sarin 2 was used by Iraq during its conflict with Iran and during the first Gulf War (Black 1993). This has led to the suggestion that exposure to sarin 2 may be a contributing factor to the so-called Gulf War Syndrome (Ember 1996). Sarin 2 was also used by terrorists in an attack on a Tokyo subway in 1995 which resulted in twelve deaths and more than 5,000 casualties (Satoh and Hosokawa 2000).

While the early development of OP's pursued differential nerve agents for military warfare, a new group of compounds came to the forefront for use as insecticides. In 1939 Paul Muller won the Nobel Prize for the creation of the organochlorine dichlorodiphenyltrichloroethane (DDT). Organochlorine compounds quickly became the most widely used pesticides around the world (Rusyniak and Nanagas 2004). The use of organochlorines as agricultural pesticides resulted in an effective pesticide with low human toxicity. With continued use however, the potential environmental impact of organochlorines led to their own demise after the realization DDT and its metabolites
bioaccumulate and biomagnify in the ecosystem and body tissue (Galloway and Handy 2003). By the 1970's, the use of organochlorines as insecticides were either legally banned or heavily restricted, resulting in an increased use and production of OPs, and eventually OPs becoming the preferential insecticide used around the world.

OP compounds represent the largest group of chemical insecticides in use today with over half of the eight billion dollar (US dollars) insecticide world market being represented by OPs (Casida and Quistad 2004). OPs have been a critical part of increasing production of many crops including corn, wheat, and cotton, as well as being an essential part of plant and lawn care in both the residential and commercial setting (EPA 1999). Additionally, certain OPs have been instrumental in the protection of public health against such mosquito-borne diseases as malaria, dengue fever, and encephalitis (EPA 1999). The demonstration by Schrader and coworkers that OP compounds exhibit insecticidal activity led to the first commercially developed OP, Bladen, in 1944 (Eto 1974). Further development of OPs as insecticides led to the production of thousands of chemically different OP compounds. Currently, approximately 150 different OPs are in use accounting for roughly 60 million pounds of OPs being applied to roughly 60 million acres of agricultural land on a yearly basis. Another 17 million pounds of OPs are used non-agriculturally for the protection of human health and livestock (EPA 1999). The structures of some common OP insecticides are shown in Figure 1.2.
Figure 1.2 Structure of some commonly used organophosphorous pesticides

Organophosphorus compounds are neurotoxic agents whose main mechanism of action is the inhibition of serine hydrolases, the most critically of which is the widely distributed nervous system enzyme acetylcholinesterase (AChE; EC 3.1.1.7) (Fukuto 1990; Carlson et al. 2000; Kardos and Sultatos 2000; Duysen et al. 2001; Ray and Richards 2001; Hong et al. 2003; Casida and Quistad 2004; Chambers and Oppenheimer 2004; Kamanyire and Karalliedde 2004; Rusyniak and Nanagas 2004). The covalent modification of the active site serine by an OP causes the inhibition of AChE, blocking the hydrolytic function of the enzyme. The acute toxic symptoms of AChE inhibition are attributed to the over stimulation of acetylcholine (ACh) receptors (generally muscarinic; mAChr) in both the nervous system and effector organs (Ma et al. 2003).
Cholinergic hyperstimulation following OP poisoning results in excessive glandular secretions and the well characterized clinical symptoms represented by the mnemonic word SLUDGE; Salivation, Lacrimation, Urination, Defecation, Gastric secretions, and Emesis (Rusyniak and Nanagas 2004). In addition to the acute symptoms of toxic OP insult, three other unique pathologies have been detailed, separate from the cholinergic effects of OPs. The “intermediate syndrome” typically originates one to three days after the resolution of the cholinergic effects, characterized by a muscular weakness which affects the limbs, respiratory muscles, and muscles of the neck. Recovery of muscle use generally happens five to eighteen days following the onset of symptoms (Rusyniak and Nanagas 2004). In response to certain OP’s, a second symptomology develops one to four weeks post-exposure, long after the cholinergic signs have subsided. This toxicity is known as organophosphate-induced delayed neuropathy (OPIDN) (Hong et al. 2003; Kamanyire and Karalliedde 2004; Costa 2006). Symptoms of OPIDN include a weakness of peripheral muscles in the hands and feet followed by a variable degree of sensory impairment. The third neurotoxic syndrome associated with OP exposure is a chronic neurological deficit that appears months to years after exposure. This syndrome, termed organophosphate-induced chronic neurotoxicity (OPICN), exhibits signs of cognitive deficits in such areas as memory, concentration, and problem solving (Damodaran et al. 2006). OP exposure has also been linked to a number of non-cholinergic illnesses including cancer, pulmonary toxicity, Parkinson’s and vision loss (Imamura T 1988; Denti 1994; Pogoda and Preston-Martin 1997; Senanayake 1998; Bhatt MH 1999; Meinert et al. 2000; Lotti and Moretto 2005).
While the clinical symptoms of OP toxicity appear to be well-understood, the cellular and molecular mechanisms of the toxicities that each OP compound propagates are far from figured out. The naïve hypothesis that all OP toxicity is caused by a similar mechanism, the inhibition of AChE, is carefully being replaced with a hypothesis that OP compounds interact with a multitude of specific molecular targets and cellular pathways that contribute to a wide variety of clinical symptoms (Costa 2006). Elucidation of the principal biochemical mechanisms and cellular pathways (cholinergic and non-cholinergic) induced by OP exposure could result in improved assessment of health risks associated with OP exposure.

A detailed discussion of OP exposure is needed to further define the limited knowledge of the mechanisms of OP toxicity. The following chapter of the thesis is divided into three parts; 1) A detailed description of organophosphates, 2) The mechanism of AChE, NTE, and serine hydrolase inhibition following OP insult, and finally, 3) a review of research involved in delineating the secondary targets (non-serine hydrolases) of OPs.
Chapter 2

BACKGROUND

The widespread use of organophosphates as insecticides plays a critical role in the production of the world's crops. It has been estimated that nearly half of all produced crops in the world would be lost to the devastating effects of insects and other pests if pesticides were not applied. Even with widespread use of pesticides within the United States, one-tenth of the crops are destroyed each year resulting in a $20 billion dollar loss (Talley 2001). OPs also play an important role as insecticides in the non-agricultural setting as well. OPs are used on lawns, gardens, flowers and even pets. Despite the recognized fact that organophosphates are neurotoxic agents, the importance of them as insecticides leads to their continued production and use. An understanding of OP chemical structure and different chemical characteristics is an important first step in the process of gaining an insight into OP action on biological systems.

Organophosphate structure and activity

The simplest representation of an OP is a phosphorus (P) atom that is tetracoordinate and pentavalent (Milesen et al. 1998). Three of the groups attached to the P are bound by a single bond while the fourth substituent is attached by a double bond. This general structure can be represented by Figure 2.1
R1 and R2 are generally alkoxy groups (R1,R2 = O-Methyl or O-Ethyl), while X is the most sensitive to hydrolysis during phosphorylation, and as such, has been termed the "leaving group". Most OP insecticides are phosphorothionates, compounds that contain a P=S (sulfur) bond. For phosphorothionates to become active, the P=S must first be converted to an oxon (P=O), resulting in the highly reactive phosphate OP (four oxygen groups bound to the phosphorous). Other subclasses of OPs include phosphorodithioates which are similar to phosphorothionates but with the leaving group oxygen replaced with a sulphur. A phosphate with two R group oxygens, a sulfur and a leaving group nitrogen (N) are phosphoramidates.

Although OPs share a common mode of action, the inhibition of AChE, they are a chemically diverse group of compounds that also exhibit a variable toxic effect. The chemical reactivity of OPs is dependent on the chemical structures and different substituents attached to the phosphorus. The electrophilicity of the phosphorus is the determining factor in reactivity and is generally determined by the chemical makeup of the leaving group, X. Structure activity relationships between different OPs have shown a direct link between the anticholinesterase activity and the reactivity of the phosphorus.
molecule (Fukuto 1990). The more electron-withdrawing groups on the leaving group that are attached to the phosphorus, the more reactive the compound becomes, while electron-donating groups on X diminish the activity of the OP. Electron withdrawing groups remove electrons from the phosphorus and make the P-X bond more susceptible to nucleophilic attack by the hydroxyl group of the serine during phosphorylation. The steric properties of the alkyl chains also influence the OP's ability to phosphorylate. Increased chain length and or increased branching of the R1 and R2 groups markedly decreases reactivity of the OP (Fukuto 1990).

**Conversion of thionates to oxons**

While high chemical reactivity (i.e. phosphorylation of serine hydroxyl moiety) of an OP is dependant on the P=O bond, most insecticides contain the P=S linkage. The P=S bond is a relatively stable bond and is not as polarized as the P=O due to the lower electronegativity of the sulphur group. The potent insecticidal activity of OPs with the P=S bond is due to the metabolic activation of the P=S into P=O by metabolic oxidation (Dauterman 1971). The metabolic transformations of OP’s have been the subject of extensive research. Fifteen years ago it was believed this reaction was mediated by the mixed function oxidases (MFO), a ubiquitous enzyme system responsible for the oxidation of xenobiotics in animals (Fukuto 1990). Further research more carefully defined the roles of these MFOs, the cytochrome P450 family of enzymes (CYPs). Although there are many gaps in the knowledge of the exact CYP involved in the reaction with each unique OP, the available data point toward a complex picture. The CYPs involved in the oxidative desulfuration of P=S to P=O show unique substrate specificities. The insecticide parathion is bioactivated to the oxon, paraoxon (PX) by the
CYP3A4/5 and CYP2C8 while diazinon is activated by CYP2C19 (Kappers et al. 2001; Mutch et al. 2003). Certain CYPs have also been shown to be involved in different transformations (non-activation reactions) of OPs such as, oxidative dearylation, oxidative deethylation, and the reduction of nitro groups on OP side chains (Costa 2006). CYPs also play an important role in the metabolic deactivation of OP's once they become oxons.

**Chemical breakdown of OPs**

The degradation of OPs occurs in both biological systems as well as in the environment. OPs as a class are a chemically reactive and rather unstable class of compounds. All OPs are tertiary esters which make them susceptible to hydrolytic degradation. Cleavage of any of the bonds can occur by both chemical and enzymatic cleavage which results in detoxification products (Fukuto 1990). OPs in the environment are readily hydrolyzed in soil, sediment, or surface water (Walker 2001). Relatively quick hydrolytic cleavage of OPs within the environment into non-toxic products is one of the main reasons OPs gained favor as insecticides over the more chemically stable organochlorines, which tend to bioaccumulate. Once a thionate (insecticide) becomes an oxon, biological degradation occurs via one or more of the following pathways, cytochrome P450 deactivation, catalytic hydrolysis, and/or non-catalytic hydrolysis.

The CYP family plays an instrumental role in the enzymatic degradation of OPs within a biological system. CYPs, which have been shown to be involved in the metabolic activation of OPs, have also been implicated in the deactivation. CYP2B6 has been shown to oxidize chlorpyrifos while also degrading parathion into the non-toxic products p-nitrophenol and diethylphosphorothioic acid (Hodgson 2003). Catalytic
hydrolysis of oxon OPs happens by way of a number of enzymes known as the phosphotriesterases, or A-esterases. Early studies by Aldridge discovered a group of enzymes that hydrolyze aromatic compounds and certain OPs such as paraoxon (Aldridge 1953). These enzymes were named A-esterases and could not be inhibited by OPs. Further research into the A-esterases and their detoxification of OPs resulted in the characterization of the A-esterase paraoxonase. Studies have shown that purified paraoxonase injected into animals increases their resistance to the toxic effects of OPs (Main 1956; Costa et al. 1990). A second group of esterases were also discovered that were important in the detoxification of OPs. However, compared to the A-esterases, these serine esterases were shown to be inhibited by OPs and were named the B-esterases. The B-esterases are unable to hydrolyze OPs, but by becoming inhibited and covalently bound to the OP, remove the OP from the plasma. Identified B-esterases include carboxylesterases, butyrylcholinesterase (BuChE), and the widely researched acetylcholinesterase (AChE). After the discovery that AChE is the primary target of OPs within a biological system, the focus of OP research was placed squarely on the mechanism and consequences of this interaction. The next section will focus on the mechanism of inhibition of AChE by OPs, as well as the inhibition of other major serine hydrolases.

**OP inhibition of AChE**

Acetylcholine (ACh) is one of the major neurotransmitters in the peripheral and central nervous system. While many other neurotransmitters are removed from the synaptic cleft by re-uptake into the cell, the majority of ACh is hydrolyzed into the inactive products choline and acetic acid by the enzyme AChE (Rotundo 1983; Massoulie
et al. 1993; Massoulie et al. 1998; Rotundo et al. 1998). AChE is one of the fastest enzymes known with an extremely high catalytic efficiency and turnover rate exceeding $1 \times 10^4$ molecules of ACh per second (Quinn 1987; Jennings et al. 2003). The active site serine located within the gorge of AChE is the main player of the hydrolysis reaction and is responsible for the enzymatic cleavage of ACh. AChE has been studied extensively for many different reasons for almost a century now. Areas of AChE research include as an enzyme, a therapeutic venue, a marker of cholinergic neurons, cell to cell interactions and as a target of pesticides and nerve agents, namely organophosphates (Rotundo 2003). Research into AChE and OPs concluded that this same active site serine is also the target of organophosphates.

The mechanism of phosphorylation of the active site of AChE can be divided into three parts. The first action is the phosphorylation (1), followed by one of two exclusive steps, either reactivation (2) (dephosphorylation), or a chemical reaction termed “aging” (3), illustrated in figure 2.2.
Figure 2.2 Inhibition of AChE by organophosphates

Organophosphates are substrates of AChE and their hydrolysis results in the phosphorylation of the active serine, effectively blocking the action of the enzyme on its physiological substrate, ACh (1). The resulting chemical bond between the phosphorus and active site hydroxyl group is much more stable than the bond between the carbonyl carbon of ACh and active site. Depending on the structure of the OP, the phosphorylation can last from a few h to several days (Gallo MA 1991). Following phosphorylation, the phosphorus-enzyme bond can be hydrolyzed by water or an oxime (2). While water is a weak nucleophilic agent, highly nucleophilic oximes such as 2-PAM (pralidoxime) can readily facilitate the reactivation of phosphorylated AChE (Chambers and Oppenheimer 2004). An alternative fate results when the phosphorus group attached to the active site is dealkylated, resulting in the loss of one of the alkyl...
groups (R1 or R2), the enzyme-OP complex becomes “aged” (3). Aging results in an OP-AChE conjugate that contains a phosphate oxyanion that is unable to regain enzymic activity. Dealkylation (aging) strengthens the phosphorus-enzyme bond and AChE is irreversibly inhibited (Mileson et al. 1998).

**Non-AChE esterase targets of OPs**

Analogous to the inhibition of AChE by OPs, other serine hydrolases can be inhibited by OPs including butyrylcholinesterase (BuChE), carboxylesterases (CE), and neuropathy target esterase (NTE), the enzyme implicated in organophosphate-induced delayed neuropathy (OPIDN). The essential feature of the serine hydrolases is the nucleophilic serine that attacks the OP (O'Neill 1981). The inhibition and subsequent dephosphorylation (or aging) of these serine hydrolases depends on the nucleophilicity, lipophilicity, and stereospecificity of the OP and the positioning and nature of the active site serine (Casida and Quistad 2004).

It was originally thought that both BuChE and the carboxylesterases (CE) did not serve an important physiological function in the body except as xenobiotic metabolizing enzymes, and that their elimination had little if any negative impacts on physiology or health (Chambers and Oppenheimer 2004). These enzymes were thought to act as prophylactics in the protection of the “important” enzyme AChE by becoming inhibited and subsequently destroying the OP compound by blocking the OP from further phosphorylation. Certain CEs have been shown to be more sensitive to OPs than AChE which would support the idea of CEs acting as prophylactics (Ray and Richards 2001). Not surprisingly, further research into the roles of BuChE and CE attributed important, specific functions to these enzymes outside of their role as prophylactics. The enzyme
BuChE has been shown to be important in the hydrolysis of choline and non-choline esters including the neurotransmitter ACh. BuChE also enhances the activity of certain proteases including trypsin and has been implicated in cellular proliferation and neurite outgrowth during neuronal development (Darvesh et al. 2003). CEs are ubiquitous enzymes that cleave carboxylic esters into their corresponding carboxylic acids and alcohols. Mammals have over 300 different CEs that have different specificities for a variety of substrates. CEs were originally believed to be important in only drug and xenobiotic metabolism but have recently been recognized for their role in the processing of endobiotic compounds (Redinbo and Potter 2005). This includes functioning in cholesterol homeostasis and fatty acid metabolism. With new roles being discovered for serine hydrolases that are important to the physiology of the cell, research into the inhibition of these hydrolases following OP exposure becomes more critical. When these hydrolases become inhibited, there is a possibility that other cellular pathways and up- or downstream targets are affected.

Following exposure to OPs, there is a clear toxicological description of the acute cholinergic crisis that occurs after with the inhibition of AChE. The clinical symptoms of OPIDN have also been fairly well documented that occur well after the cholinergic crisis. Research into the mechanism of OPIDN postulated that the inhibition (and subsequent aging) of a novel enzyme, neuropathy target esterase (NTE) by certain OPs is the cause of OPIDN (Barrett et al. 1985). Yet, while NTE has been implicated as the initiator of OPIDN following its inhibition, the physiological role of NTE and its involvement in the manifestation of OPIDN have yet to be fully characterized. NTE has been shown to have a physiological role in lipid metabolism and homeostasis (Zaccheo et al. 2004). The
phosphorylation and aging of NTE happens within hours of exposure, yet the clinical symptoms of OPIDN manifest 2-4 weeks afterwards. The clinical symptoms attributed to OPIDN begin with a tingling and loss of sensation in the hands and feet. Cramping pains in the legs ensue, followed by rapidly progressive muscle weakness of the lower limbs. Severe cases include loss of the ability to coordinate muscle movements and eventual paralysis of lower and upper extremities. Patients may eventually regain function of limbs with diminished operation (Rusyniak and Nanagas 2004). Pathological studies after NTE inhibition but before clinical symptoms, demonstrated degeneration of axons and their terminals as well as spinal cord atrophy. The deterioration of the peripheral axons is directly proportional to the length of the specific axon (Rusyniak and Nanagas 2004). Interestingly, the symptoms of OPIDN appear with only a select group of OP’s. OPIDN symptoms are also unrelated to the cholinergic and intermediate symptoms, occur well after those symptoms have subsided, and cannot be attributed to the inhibition of AChE, which occurs with every type of OP compound (Costa 2006). With the growing body of research on NTE and OPIDN, the mechanism and pathways from the progression of NTE inhibition to axonal degradation to clinical symptoms of OPIDN remain undefined.

Additional non-AChE, non-NTE syndromes

The clinical symptoms of the “intermediate syndrome” following OP exposure, which are characterized by a weakness of respiratory, neck and proximal limb muscles, have not been directly correlated with a molecular target. The signs appear after cholinergic resolution but before OPIDN, yet the only hypothesis concerning the effects of the intermediate syndrome is cholinergic receptor desensitization due to prolonged
cholinergic stimulation (Lotti 2001). Again, the exact mechanism of the syndrome is unclear.

To further confound the research of mechanisms of symptoms associated with OP exposure, is the somewhat controversial research studying the pathology of the chronic neurological conditions persisting long after the OP insult. Studies have shown different cognitive effects following OP exposure including deficits in memory, problem solving, and concentration (Rusyniak and Nanagas 2004). There have also been reports of psychiatric problems such as depression and anxiety. However, not all groups studying cognitive effects following OP exposure have shown the same associations. With this multitude of research describing different clinical syndromes associated with OP poisoning, and the firm belief that these symptoms cannot be adequately explained solely by the inhibition of AChE or NTE, scientific studies began to investigate secondary targets and pathways affected by OP compounds.

Secondary targets of OP exposure

It is difficult to make generalized comments about the mechanistic action of OPs on biological systems outside the inhibition of AChE. Many studies have been done on different model systems with a wide array of different OPs at all types of concentrations resulting in a multitude of responses. Because there are many different OPs and concentrations used in OP research, it is important to not make a broad generalization about all OPs when discussing secondary targets. However, there is a significant quantity of research that points to the fact that OPs do affect secondary targets and pathways within the cell. The next section will discuss four different broad groups of targets presented here as secondary targets of OPs. The first section will be unique DNA /
mRNA / protein products that are modulated, altered, or blocked by OPs. The next section will look at certain well established pathways in the cell affected by OPs. The affect of OPs on both the immune system and cognitive behavior will make up the last two parts of the discussion.

**DNA:** Certain OP methyl esters have been shown to damage DNA through methylation. This alkylation of DNA can cause point mutations within the DNA strand, opening the strand to possibilities of mismatched pairs. The OP trichlorfon was demonstrated to inhibit the enzyme O\(^6\)-methylguanine-DNA methlytransferase which is involved in the catalytic repair of O\(^6\)-methylguanine (Badawi 1998). Inhibition of this enzyme further complicates the effect of DNA alkylation.

**Gene expression changes:** Cholinergic gene expression changes are generally an indirect effect resulting from increased levels of ACh following AChE inhibition by OPs. Expression levels of AChE mRNA was monitored at different time-points following the injection of the nerve agent Sarin (0.5\(\times\) LD\(_{50}\)) into rats (Damodaran et al. 2003). AChE showed differential expression in different regions of the brain following sarin treatment in comparison to control. Two hours following treatment, AChE was induced almost three-fold with a return to basal level by 24h.

Mouse brain slices exposed to diisopropylfluorophosphate (DFP) showed overexpression of c-fos (early response transcription factor), choline acetyltransferase (ChAT), and vesicular ACh transporter (VACHT) (Kaufer et al. 1999).

**OPs are able to directly inhibit steroid hormone levels by blocking adrenocorticotropin and cAMP-stimulated steroidogenesis (Given et al. 1977).** More recent investigations indicate OPs may block steroidogenesis by blocking transcription of
the steroidogenic acute regulatory (StAR) gene (Walsh et al. 2000). The StAR protein mediates the rate-limiting step in steroidogenesis and its decreased expression can lead to impaired reproductive function.

**Protein modulation:** The response of cholinergic receptors, both muscarinic (mAChR) and nicotinic (nAChR), has also been studied in response to OPs. Because of the different model systems (*in vitro* and *in vivo*) and different types and concentrations of OPs used, it is difficult to make a generalized summary about the effects of OPs on cholinergic receptors. Nevertheless, OPs directly modify cholinergic receptor activity (activating and blocking), downregulate receptor number (both directly and indirectly through the inhibition of AChE), and cause differential regulation of both nAChR and mAChRs mRNA (directly and indirectly) (Jett et al. 1993; Khan et al. 2000; Casida and Quistad 2004).

The phosphorylation (activation) of Ca\(^{2+}\)/cAMP response element binding protein (CREB) was shown to occur following low dose exposure (doses that do not inhibit AChE) of primary cortical neurons with chlorpyrifos (Schuh et al. 2002). CREB is an important activator of gene transcription and is involved in the control of many cell processes, including hormone synthesis in endocrine cells and the formation of long-term memory in neurons (Alberts 2002).

The nerve agent Soman was shown to directly inhibit the release of norepinephrine from rat brain cortical slices and was suggested that it was acting through direct action at NMDA receptors (Tang and Cassel 1998).

High non-lethal doses of malathion induced variable amounts of cell rounding and shrinkage as well as progressive loss of cell-cell and cell-substrate adhesion in cultured
breast cancer cells (Cabello et al. 2003). This toxicological phenotype was found to be due to the decreased expression of two different epithelial adhesion proteins, E-cadherin and B-catenin. At lower concentrations of malathion, increased expression of two small GTPases (Rho and Rac1) were measured.

**Cellular pathways:** OPs have been demonstrated to alter cellular pathways. Calcium (Ca\(^{2+}\)) is involved in many different aspects of the cell including functioning as a ubiquitous intracellular messenger and integral part of neurotransmission. Ca\(^{2+}\) homeostasis within the cell is an important part of signaling pathways in the cell and is directly modified by OPs. Sun et al. showed low levels of PX (0.1 nM-10 nM) exposure significantly increased basal levels of Ca\(^{2+}\) in human parotid salivary cells (HSY) (Sun et al. 2000). Pre-exposure of the HSY cells to nanomolar levels of PX also increased Ca\(^{2+}\) release from 1,4,5-inositol-triphosphate (IP\(_3\))-sensitive stores following addition of a muscarinic agonist, suggesting PX increases the sensitivity of IP\(_3\) receptors (Sun et al. 2000). It was hypothesized that PX is acting directly on these downstream targets of the phosphoinositide signaling pathway.

High concentrations (mM) of PX have been shown to cause a transient increase in intracellular Ca\(^{2+}\) in SK-N-SH-SY5Y cells (Hong et al. 2003). However, this same increase was shown with the hydrolysis products of PX as well. Yet, repeated doses of 50 \(\mu\)M PX decreased the basal level of intracellular calcium over time (Hong et al. 2003). Clearly PX affects different mechanisms involved in calcium signaling pathways and the homeostasis of intracellular calcium.

PX has also been demonstrated to induce apoptosis. A mouse T-lymphocytic leukemia cell line EL4 was exposed to 10 nM PX for 16 h. Employing a combination of
specific caspase inhibitors, it was shown that PX induced apoptosis by disruption of the mitochondrial transmembrane potential, which in turn caused the release of cytochrome \( c \) into the cytosol and subsequent activation of caspase-9 (Saleh et al. 2003). Caspase-9 then cleaved the effector caspase-3 which began the downstream events of apoptosis.

OPs have also been implicated in producing reactive oxygen species (ROS) which induces oxidative stress and a cellular response. ROS are either free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can produce free radicals or are chemically activated by them. The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. Lipid peroxidation has been suggested as the molecular mechanism involved in OP oxidative stress (Khrer 1993). When oxidative stress occurs, a large number of different ROS scavenging enzymes are released to remove the oxidative free radicals. Clinical studies of malathion-exposed patients detected increased levels of free radical scavenging enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and gamma glutamyl transpeptidase enzymes in blood samples in comparison to control (Banerjee et al. 1999; Seth et al. 2001). OP induced lipid peroxidation has also been demonstrated in rat brains and human erythrocytes (Gultekin 2000; Verma 2001).

Immune responses: The elimination and/or inactivation of foreign bodies in a biological system are performed by the immune system. The immune system is composed of adaptive (have memory) and innate (no memory) elements (Alberts 2002). Innate immune responses are not specific to a particular pathogen and rely on proteins and phagocytic cells to recognize conserved features of pathogens and become quickly
activated to help destroy invaders. The adaptive immune response is a more specialized response that has the ability to recognize and remember foreign pathogens and synthesize antibody molecules that react specifically to destroy or inactivate the stimulating pathogen (antigen). There is a large body of evidence that illustrates the capability of OPs to cause immunotoxicity, which is defined as an adverse effect on the immune system or its component parts. Research using animal models has demonstrated two general characteristics of the immune response after OP exposure. Acute doses to animals that cause cholinergic toxicity show decreased immune function within the animal. Low doses of OPs that do not inhibit AChE, produce enhanced immune function (Pruett 1992). While it is generally difficult to identify the exact molecular targets and mechanisms of OP immunotoxicity, it is most likely a combination of immune system targets that elicits the immunotoxic effect. Serine hydrolases are involved in the thrombin systems of the immune system and different esterases are associated with the cellular membranes of immune system lymphocytes and monocytes (Stepanovic 1998). The inhibition of these serine hydrolases and esterases could potentiate the effects of OP-induced immunotoxicity (Galloway and Handy 2003). OPs could also possibly cause oxidative stress to lymphoid tissues or different immune organs. Indirect effects on the immune system could be propagated by altered cholinergic output to different lymphoid organs as well as a chronic OP toxicity causing changed metabolism followed by malnutrition and subsequent immunodepression (Galloway and Handy 2003).

The cytokine IL-1β is one of several cytokines involved in inflammation and the immune response. Elevated levels of IL-1β are associated with both injury and illness. Rats injected with soman (1 x LD₅₀), showed increased IL-1β mRNA and protein levels
30 min and 6 h, respectively, post-exposure (Svensson et al. 2001). The highly reactive nature of OPs is able to induce a multitude of different effects within an organism to cause immunotoxicity.

Cognitive defects: Long-term cognitive deficits have also been shown to be associated with OP exposure. The cessation of cholinergic toxicity following OP insult can be followed by a so called “intermediate syndrome”, OPIDN, or the possibility of some type of neurological cognitive deficit. This third type of neurotoxic action of OPs has just recently been named organophosphate-induced chronic neurotoxicity (OPICN) (Damodaran et al. 2006). OPICN symptoms have been characterized in response to large and sub-clinical doses of OPs. Specific examples of OPICN have occurred in victims of the sarin attacks on the subways of Tokyo and in veterans of the first Gulf War who were suspected of low level exposure to sarin (Damodaran et al. 2006). While no specific targets have been linked, there are many clinical cases of victims of OP exposure that show a definite cognitive defect. The results from several studies have demonstrated the presence of OP-induced learning impairments, loss of concentration, depression, and difficulty in forming memories (Prendergast et al. 1998; Stone et al. 2000; Rusyniak and Nanagas 2004). The pathology of these cognitive deficits has yet to be clearly demonstrated; current theories include the down regulation of either cholinergic and non-cholinergic receptors (cholinergic receptors involved in memory formation), neuronal cell death, or some other unidentified brain proteins affected by OPs (Rusyniak and Nanagas 2004).
Summary

The reaction of OPs with AChE and the cholinergic toxicity that follows is fairly well characterized. In general the neurotoxicity post OP insult is attributed to the inhibition of AChE. However, three other developed clinical syndromes have been attributed to OP exposure and cannot solely be explained by AChE inhibition. The so-called “intermediate syndrome”, organophosphate-induced delayed neuropathy (OPIDN), and the neurotoxic cognitive deficit OPICN have been the subject of different studies, yet the precise mechanism and pathways affected that bring about the clinical symptoms are still unclear. The characterization of the mechanism of OPs phosphorylating the active site serine of AChE, brought about the realization that OPs might also be targeting secondary biomolecules in the cell including other serine hydrolases and esterases, as well as yet unidentified protein and/or pathways. Further investigations into secondary targets of OPs uncovered a number of different non-AChE targets within the cell, including cholinergic receptors and other specific protein products (as described above). However, the discovery of these secondary targets did little to further the understanding of non-cholinergic toxicity following OP insult. A clearer understanding of cellular pathways affected by OPs and the different mechanisms by which OPs cause toxicity is needed.

The wide use of OPs as insecticides in both the agricultural and urban setting will continue for the foreseeable future (Costa 2006). OPs are relatively inexpensive and have low bioaccumulation within the ecosystem. However, because OPs are not selective, human exposure is of concern and research into the targets and mechanisms of OP
toxicity outside of cholinergic hyperstimulation is desirable. The work introduced in this study begins to address these current issues in organophosphate toxicology. Initially, an analysis of gene expression changes in response to an acute, low-dose of the organophosphate paraoxon was performed using cDNA microarray and SH-SY5Y human neuroblastoma cells. The next phase of research involved confirming gene expression changes using more traditional techniques such as RT-PCR and immunoblot. Following confirmation of genes, gene data from the microarray experiments were organized into groups designated by a set of genes involved in specific cellular pathways. Additional experiments were devised to test the modulation of these pathways by paraoxon.
Chapter 3

OBJECTIVES / SPECIFIC AIDS

1. Identify and compare reproducible changes in gene expression following an acute, low dose, organophosphate (paraoxon (PX), vehicle) exposure to a human neuroblastoma cell line, identified by cDNA microarray with an in-house human tox cDNA chip.

2. Confirm a select gene expression (genes involved in a certain cellular pathway such as apoptosis or proliferation) change from Aim 1 by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), protein analysis, and/or enzyme activity assay.

3. Experimentally test the hypothesis that a specific cellular pathway (identified from microarray data in Aim 1) has been affected by exposure to PX.
Chapter 4

METHODS AND MATERIALS

Cell Culture:

SH-SY5Y human neuroblastoma cells (ATCC) were maintained in DMEM/F-12 (Dulbecco's Medium: Ham's F-12, 1:1 mix) medium supplemented with 10% (v/v) fetal bovine serum (FBS), 60mg/l (100 U/ml) penicillin and 100 mg/l streptomycin, in a Forma Scientific Incubator at 37° C in a humidified atmosphere of 95% air, 5% CO₂. Cells were grown in monolayer culture and harvested at confluence for further passage by standard cell culture techniques. Cells were harvested using 0.05% trypsin/0.53 mM EDTA in HBSS.

Paraoxon (PX) Treatment:

Treatment was performed on SH-SY5Y cells in passages 4-8. Paraoxon (98.6% purity) was purchased from ChemService (West Chester, PA) and was diluted to working concentrations in acetone. Cells were treated with either 10 nM PX or acetone (0.1%) alone as a vehicle control.
Microarray Analysis:

**RNA purification:** SH-SY5Y cells were passed into T-75 flasks at a density of 1x10^7 cells/flask and allowed to incubate 48 h prior to treatment. Cells were exposed to 10 nM PX or acetone control and RNA was harvested at three specific time-points- 3, 8, and 24 h. Each PX-treated flask had a corresponding control flask treated with acetone for the exact same time. Cells were harvested by scraping and RNA extracted and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA). Following purification of the RNA, spectrophotometer measurements (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE) were made and only RNA with a 260/280 ratio of 1.9-2.1 was used in the microarray analysis.

**Conversion of RNA to fluorescently labeled cDNA:** Conversion of the RNA to fluorescently labeled cDNA was carried out using the SuperScript Plus Direct cDNA Labeling System (Invitrogen, Carlsbad, CA). Briefly, 10 μg of RNA was incubated with 5 μg of Oligo(dT)_{20} primer at 70°C for 10 min and then placed on ice. Following incubation, the RNA-Oligo mix was added to First-Strand buffer (final concentration= 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂), 0.01 M DTT, Nucleotide Mix (mixture of dCTP, dGTP, dATP, dTTP, and labeled dUTP in 10 mM Tris (pH 8.0), 1 mM EDTA), 40 U of RNaseOUT, and 800 U of SuperScript III RT. PX-treated RNA was incubated with Alexa Fluor 647-aha-dUTP Nucleotid Mix while the control RNA was incubated with Alexa Fluor 555-aha-dUTP Nucleotide Mix, for three h at 46°C. The
labeled cDNA was then purified and checked for labeling efficiency using the Nanodrop spectrophotometer.

Hybridization: Analysis was performed for each time-point in duplicate using an in-house Human Toxicology cDNA Microarray Chip (version 3, Dr. Mark Pershouse Lab, University of Montana, Missoula, MT) that contained approximately 1600 unique cDNA’s (each slide printed with 6 replicate spots of each gene) of genes generally implicated in a toxic insult. Labeled probes were denatured for 2 min at 95°C, snap cooled on ice, and then added to the Human Tox microarray chip and allowed to hybridize overnight at either 42°C or 50°C. The hybridized slides were quickly agitated in 1X SSC, 0.01% SDS, followed by a) 2X two min washes in 1X SSC, and b) 2X two min washes in 10 mM TE. Washed slides were quickly dried by centrifugation and stored in a dark box until they were ready to be scanned.

Scanning and Data Analysis: Hybridized microarray slides were scanned using the Gene Pix 4000B Scanner (Axon Instruments) and the Gene Pix Pro 5.1 software (Axon Instruments). The data from each scanned digital image was analyzed using Iobion Gene Traffic (Iobion Stratagene Analysis Software, Iobion Informatics LLC, Stratagene, La Jolla, CA). Initially, data was normalized to a total 1:1 red to green ratio for the entire slide. Data were then filtered based on replicate quality, signal intensity, and signal-to-background ratios of 1.2 or higher. An arbitrary cutoff of 1.5 fold upregulation and 1.4 fold downregulation was used to select genes of interest.
Semi-Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR):

RNA purification was carried out following the same protocol used for the microarray analysis. cDNA synthesis of the RNA was done according to the protocol provided in the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen). Briefly, 2 μg of RNA was combined with 5 μM oligo(dT)$_{20}$, 1mM dNTP mix and incubated at 65°C for 5 min followed by 1 min on ice. With the tube on ice, the following were added: RT buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 5 mM MgCl$_2$, 10 mM DTT, 40 U RNaseOUT, and 200 U SuperScript III RT. After gentle mixing, reactions were incubated for 50 min at 50°C. Reactions were terminated by heating at 85°C for 5 min and then placed on ice. 2 U of RNase H were added to each reaction and allowed to incubate for an additional 20 min at 37°C. cDNA synthesis reactions were either used for PCR immediately or stored at -20°C.

PCR reactions were carried out in a programmable PTC-200 (Bio-Rad, Hercules, CA) thermal cycler using Platinum PCR SuperMix (Invitrogen) while following the accompanying protocol. ECM1, ODC1, and TUBG1 primers were designed using PrimerQuest software from Integrated DNA Technologies, Inc. β-actin primers were used to amplify actin as an internal control (Maxim Biotech, Inc., South San Francisco, CA), and were expected to yield a product of 303 bp. Briefly, reaction tubes contained 10 ng of cDNA template, 500 nM of selected primers, and 45 μL of Platinum PCR SuperMix (1U complexed recombinant Taq DNA polymerase with Platinum Taq Antibody, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl$_2$, 200 μM dGTP, 200 μM dATP, 200 μM dTTP, and 200 μM dCTP). The PCR reactions were cycled as follows: 2
min at 94°C to activate Taq (1 cycle); 30 sec at 94°C (denaturation step), 30 sec at 62° (annealing step, temperature used for all primer sets) and 1 min at 72°C (extension step) for 30 cycles (all primer sets).

After the PCR reaction, PCR products were electrophoresed alongside a 100 bp DNA ladder (Promega, Madison, WI) through a 1% (w/v) agarose (Bio-Rad) gel stained with ethidium bromide. Images were captured using a Kodak DC290 digital camera and optical density values of PCR products were quantified using Quantity One software v.4.2.1 (Bio-Rad). PCR reactions were done in triplicate and statistical analysis was done with Prism v4.0 software.

**Immunoblot analysis:**

Treated SH-SY5Y cells were removed by scraping and subsequently lysed for 30 min on ice in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% (v/v) Triton X-100). Lysate was centrifuged shortly to pellet heavy membranes. Protein concentration of the lysate was determined by the BCA method (Pierce, Rockford, IL). All immunoblots were done in triplicate.

**ECM1 immunoblot**- 15 µg of protein was loaded onto a 10% PAGE gel (Bio-Rad), electrophoresed and transferred to PVDF membrane (Bio-Rad). The membrane was, a) blocked with 2% BSA (Calbiochem, La Jolla, CA) in phosphate buffer saline with Tween (PBS-T, pH 7.4, Tween 0.1%) for 1 h at room temperature, b) washed in PBS-T for 10 min, c) incubated with rabbit anti-ECM1 (1:1000 in block) overnight at 4°C, d) washed
2X quickly, 1X 15 min in PBS-T, e) incubated with donkey anti-rabbit HRP linked (1:10,000 in block) for 1 h at room temp, f) washed 2X quickly, 1X 15 min in PBS-T, and finally, g) visualized with ECL Plus Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ) using a Fujifilm LAS-3000 imaging system (Fuji Photo Film, Valhalla, NY) and Image Gauge v4.22 software.

**ODC1 immunoblot-** Followed exact same protocol as ECM1 Western, with a few exceptions. 30 µg of lysate was loaded in each lane and the primary antibody, goat anti-ODC1 (1:500 in block) was incubated for 1 h at room temp. The secondary antibody was horse anti-goat (1:2000 in block).

**Ornithine Decarboxylase (ODC) Activity Assay:**

ODC activity was quantified using a modification of the procedure described by Coleman and Pegg (Morgan 1997). Cell extracts were lysed in a solution of 10 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol (DTT), and 0.1 mM EDTA (250 µL). Cells were frozen and thawed three times followed by centrifugation at 12,000 x g for 15 min at 4°C and then placed on ice. 100 µL of supernatant was combined with 100 µL of lysis buffer and a 50 µL aliquot of assay mix (12.5 µL of 1M Tris-HCl, pH 7.5, 5 µL of 2 mM pyridoxal 5’-phosphate, 2.5 µL of 250 mM dithiothreitol, 5 µL of 20 mM L-ornithine, 2.5 µL of L-[1-14C] ornithine, and 22.5 µL of deionized water) in a glass assay tube. The reaction tube was immediately closed with a rubber stopper containing a center well holding a quarter piece of a 5.5 cm Whatman filter paper soaked in 1 M NaOH. Tubes
were allowed to incubate in a shaking water bath for 1 h at 37°C and the reaction was then stopped by adding 0.25 mL of 5 M sulfuric acid through the rubber cap. After a further 1 h incubation, the filter paper was removed and placed in 3 mL of scintillation liquid plus 25 μL of 17 M glacial acetic acid (to reduce lumex number) and counted in a scintillation counter. 30 μL of remaining cell lysate was TCA precipitated and then subject to BCA assay to determine protein concentration. Data were calculated as nanomoles of CO₂ per milligram protein per hour.

**Cell Proliferation Assays:**

**Cell Counting-** SH-SY5Y cells were passed into a 6 well plate at 1x10⁵ cells/well and allowed to adhere for 48 h. Cells were exposed to 10 nM PX or acetone control in triplicate. Cells were allowed to incubate for 6 days before harvesting. At 6 days post treatment, media was removed, cells were washed in PBS, and next, 0.75 mL of trypsin was added to each well and plate was incubated for an additional 5 min. 0.75 mL of PBS was added to each well and the contents of each well were removed and placed in a microcentrifuge tube. 35 μL of cell/PBS/trypsin solution was combined with 35 μL of trypan blue, mixed, placed on a hemacytometer, and counted. Three separate areas of the hemacytometer were counted and recorded for each well.

**MTT Assay-** SH-SY5Y cells were passed into a 96 well plate at 2 different densities (1 and 3x10⁴) in 200 μL of media/well and allowed to adhere for 48 h. Two different concentrations of PX (10 nM and 1 nM) or acetone (control) were added to the cells. For
each cell density and each concentration of PX, 3 wells of cells were treated along with 3 wells of acetone-treated cells, for a total of 24 wells treated per experiment. For each cell density, the different concentration of PX-treated cell counts were compared to the total acetone treated cell counts (3 wells of PX vs. 6 wells of acetone for each cell density). At 96 h post PX treatment, the MTT assay (Roche, Indianapolis, IN) was performed using the accompanying protocol. Briefly, 20 μL of MTT labeling reagent (final concentration 0.5 mg/mL) was added to each well. The plate was incubated in a humidified incubator (same as cell culture above) for 4 h following labeling. Next, 100 μL of Solubilization solution was added to each well and the plate was again placed in the humidified incubator overnight. The plate was read on a microplate reader at 560 nm with a reference wavelength of 690 nm.

Materials:

Cell culture media was purchased from ATCC (Manassas, VA). FBS, antibiotics, and trypsin were purchased from Invitrogen. Trypan blue, EDTA, L-ornithine, and pyridoxal 5'-phosphate were all obtained from Sigma (St. Louis, MO). L-[1-14C] ornithine hydrochloride was purchased from GE Healthcare. The anti-ECM1 polyclonal antibody was provided by Professor Lurong Zhang at the University of Rochester. Anti-ODC antibody was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA).
Chapter 5

Transcriptional changes in the SH-SY5Y human neuroblastoma cells following acute, low-dose exposures to the organophosphate paraoxon

Introduction

Microarray technology allows the rapid and quantitative analysis of gene expression patterns within a cell or area of tissue. Microarray allows researchers to survey gene expression changes of entire genomes in different species including human, mouse, and yeast. The use of microarray technology in experiments has been applied to many different areas of research including genotyping, development, human disease, drug discovery, and notably, the field of toxicology (Afshari et al. 1999; Schena 2003; Newton et al. 2004). Within the field of toxicology, microarray is used as a screening tool for the identification of molecular targets and mechanisms of toxicity. A review by Vrana et al. states that microarray "enables researchers to identify those genes and their products (either single or whole pathways) that are involved in conferring resistance or sensitivity to toxic substances" (Vrana et al. 2003).

The SH-SY5Y human neuroblastoma cell line, with an adrenergic neuronal phenotype and expression of relatively high levels of AChE and NTE, has been used in numerous toxicological studies as well as a model system for OP toxicity testing (Ehrich 1995; Veronesi et al. 1997; Barber and Ehrich 2001; Hong et al. 2003). Gene expression patterns have also been characterized in SH-SY5Y cell lines using microarray, following treatment with a wide variety of compounds including ethanol, nicotine, tunicamycin (an inhibitor of glycosylation), and the Parkinson’s disease causing metabolite MPP⁺ (1-
The work presented in this chapter details the transcriptional changes in human neuroblastoma cells (SH-SY5Y) following a time-course of acute, low-dose exposures to the organophosphate paraoxon, measured by cDNA microarray. Data analysis of genomic changes was further examined using the GoMiner software (available at http://discover.nci.nih.gov/gominer/). GoMiner is a tool used to gain insight into the biological relevance of transcriptional changes within an experiment. The findings presented in this chapter will help to begin to understand and define the pathways and different cellular targets modified by OP exposure.

Results

To assess the extent to which the OP paraoxon (PX) modulates gene expression in neuronal cells, transcriptional changes were monitored using an in-house, Human Toxicology microarray chip (Mark Pershouse Laboratory, University of Montana) containing approximately 1600 different genes. The genes represented on the Human Tox chip have defined functions in basic cellular processes as well as roles in specific responses to different types of toxic insult. While these genes are not representative of the entire genome, they do provide a foundation for certain pathways or targets affected by OPs. RNA was isolated from SH-SY5Y neuroblastoma cells treated for 3, 8, and 24 h with 10 nM PX or acetone vehicle. Previous experimental data using OPs has come under criticism for the unrealistically large amount of OP that was needed to observe any
significant change in protein levels (Ray and Richards 2001). The documented cases of OP poisoning usually exhibit some degree of cholinergic toxicity, but there are a significant number of undocumented cases that are exposed to substantially lower concentrations of OP. Although concentrations of OPs in the blood may reach micromolar range following a severe poisoning, the target tissues and cellular proteins and pathways being perturbed by the OP are most likely due to much lower concentrations (Sun et al. 2000). The fact that OPs show low environmental persistence due to biodegradation has led to the belief that many of the toxic effects of OPs are due to acute rather than chronic exposures (Casida and Quistad 2004). It was for these reasons that a low-dose (10 nM), acute (3, 8, 24 hr) exposure of PX was used.

The use of cDNA as targets on the microarray chip affords greater ability to observe low abundance mRNA’s. However, with the long length of the cDNA probes compared to oligo probes, what is made up in targeting lower abundance mRNA’s is lost in specificity. A higher hybridization temperature creates more specificity between target and probe, yet reduces the amount of probe-target hybridizations. For this reason, fluorescently labeled cDNA was hybridized with the chip at two different temperatures, 42° and 50°, each done in duplicate, for a total of four microarray slides per time-point. On the Human Tox microarray chip, each gene is spotted six times on the slide, with two different areas being spotted, and each area done in triplicate. After normalization and background reduction, genes of interest were selected using an arbitrary cut-off of at least 1.5 fold or greater up-regulation, 1.4 fold or greater down-regulation, and at least 5 or more valid spots of the 24 total spots. While a total of 24 spots were available for each gene, the combination of some genes not hybridizing at the higher temperature (loss of 12

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spots) and the loss of occasional valid spots due to user manipulation during the experiment resulted in the use of 5 or greater valid spots as the cut-off. Microarray data analysis revealed a total of 15, 49, and 26 genes up-regulated after 3, 8, and 24 h of 10 nM PX treatment using these filters, respectively. The number of down-regulated genes compared to control was 6, 9, and 7 for each successive time-point. Table 5.1 provides a list of differentially expressed genes following PX exposure.

Table 5.1 Up- and downregulated genes in SH-SY5Y cells after exposure to 10 nM PX

| Genes marked with a (3,8) showed up- or downregulation at the 3 and 8 h time points |
| Genes marked with a (8,24) showed up- or downregulation at the 8 and 24 h time points |
| Genes marked with a (3,8,24) showed up- or downregulation at all time points (3, 8, 24 h) |
| Genes marked with a (3,24) showed up- or downregulation at the 3 and 24 h time points |
### 3 h 10 nM PX Upregulated genes

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Valid Spots</th>
<th>Fold Change</th>
<th>STDEV</th>
<th>Time Points</th>
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<tr>
<td>R34002</td>
<td>ISGF3G</td>
<td>5</td>
<td>2.2</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>AA099195</td>
<td>GDI2</td>
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<td>2</td>
<td>0.34</td>
<td></td>
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<tr>
<td>AA033966</td>
<td>CYP2C8</td>
<td>11</td>
<td>1.9</td>
<td>0.43</td>
<td>(3,8,24)</td>
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<tr>
<td>R72075</td>
<td>NRG1</td>
<td>6</td>
<td>1.8</td>
<td>0.6</td>
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<td>N62663</td>
<td>KDELR</td>
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<td>1.7</td>
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<tr>
<td>AA04204</td>
<td>COL5A2</td>
<td>9</td>
<td>1.65</td>
<td>0.31</td>
<td>(3,8)</td>
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<td>R78620</td>
<td>CXCR4</td>
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<td>0.58</td>
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<td>W44542</td>
<td>GNAI1</td>
<td>13</td>
<td>1.6</td>
<td>0.44</td>
<td>(3,8,24)</td>
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<td>AA029292</td>
<td>OXTR</td>
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<td>1.5</td>
<td>0.21</td>
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<td>AA100835</td>
<td>RAP80</td>
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<td>1.5</td>
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<td>AI381503</td>
<td>IL5RA</td>
<td>7</td>
<td>1.5</td>
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<td>H44575</td>
<td>DST</td>
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<td>1.5</td>
<td>0.25</td>
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<td>N79484</td>
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<td>1.5</td>
<td>0.25</td>
<td>(3,8,24)</td>
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<td>GPR65</td>
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### 3 h 10 nM PX Downregulated genes

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<th>Valid Spots</th>
<th>Fold Change</th>
<th>STDEV</th>
<th>Time Points</th>
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<tr>
<td>AA045178</td>
<td>LIG1</td>
<td>9</td>
<td>-1.7</td>
<td>0.12</td>
<td>(3,8,24)</td>
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<tr>
<td>AA043211</td>
<td>ACSL1</td>
<td>9</td>
<td>-1.5</td>
<td>0.49</td>
<td></td>
</tr>
</tbody>
</table>

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AA242743  XPA  8  -1.5  0.21  (3,24)  
*Xeroderma pigmentosum, complementation group A*

BG572956  BIRC2  9  -1.5  0.36  (3,24)  
*Baculoviral IAP repeat-containing 2*

AA046463  DHPS  9  -1.4  0.19  
*Deoxyhypusine synthase*

AA028080  AKT3  9  -1.4  0.2  
*V-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)*

**8 h 10 nM PX Upregulated genes**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Valid Spots</th>
<th>Fold Change</th>
<th>STDEV</th>
<th>Time Points</th>
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<tr>
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<td>ODC1, Ornithine decarboxylase 1</td>
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<tr>
<td>AA029766</td>
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<td>2.3</td>
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<td>(8,24)</td>
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<tr>
<td>R46574</td>
<td>FCGR2A, Fc fragment of IgG, low affinity IIa, receptor (CD32)</td>
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<td>0.31</td>
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<td>GNAI1, Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1</td>
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<td>W94612</td>
<td>MCM2, MCM2 minichromosome maintenance deficient 2, mitotin (S. cerevisiae)</td>
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<td>N79484</td>
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<th>Log Fold Change</th>
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<th>Gene ID</th>
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8 h 10 nM PX Downregulated genes

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<td>CXCLI3</td>
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**24 h 10 nM PX Upregulated genes**

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<tr>
<td>AA029766</td>
<td>TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kDa</td>
<td>21</td>
<td>1.8</td>
<td>0.3</td>
<td>(8,24)</td>
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<td>Glutamate receptor, metabotropic 3</td>
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<td>R46574</td>
<td>Fc fragment of IgG, low affinity IIa, receptor (CD32)</td>
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**W44542**  
GNAI1  
17  
1.5  
0.25  
(3,8,24)  
Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1

**AA010576**  
DHFR  
21  
1.5  
0.17

Dihydrofolate reductase

**AA033966**  
CYP2C8  
20  
1.5  
0.3  
(3,8,24)  
Cytochrome P450, family 2, subfamily C, polypeptide 8

**AA046892**  
DAD1  
24  
1.5  
0.09

Defender against cell death 1

**AA056507**  
IFRD1  
22  
1.5  
0.22

Interferon-related developmental regulator 1

**AA059066**  
ZF  
21  
1.5  
0.19

HCF-binding transcription factor Zhangfei

**H57126**  
CSFR1  
24  
1.5  
0.2

Colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog

**W74060**  
NR1D2  
24  
1.5  
0.11

Nuclear receptor subfamily 1, group D, member 2

**W76604**  
RAB28  
8  
1.5  
0.16

RAB28, member RAS oncogene family

**W78013**  
MCM3  
18  
1.5  
0.32

MCM3 minichromosome maintenance deficient 3 (S. cerevisiae)

---

**24 h 10 nM PX Downregulated genes**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Valid Spots</th>
<th>Fold Change</th>
<th>STDEV</th>
<th>Time Points</th>
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<tbody>
<tr>
<td>AA410383</td>
<td>CXCL13  Chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)</td>
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<td>R37866</td>
<td>IQGAP1 IQ motif containing GTPase activating protein 1</td>
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<td>(8,24)</td>
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<td>AA045178</td>
<td>LIG1    Ligase I, DNA, ATP-dependent</td>
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<td>-1.5</td>
<td>0.12</td>
<td>(3,8,24)</td>
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<td>AA242743</td>
<td>XPA     Xeroderma pigmentosum, complementation group A</td>
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<tr>
<td>BG572956</td>
<td>BIRC2   Baculoviral IAP repeat-containing 2</td>
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Initial microarray data analysis

The data in Table 5.1 represents the list of genes whose expression were considered significantly altered following PX exposure for 3, 8, and 24 h. The validity of each gene was initially assessed on the number of valid spots on each of the replicate microarray slides. Further analysis was done on each gene by observing the individual spots that makeup the data for that gene, including number of valid spots, fold change, and standard deviation (STDEV). The STDEV for each gene was computed using the fold changes of each valid spot, however, if one or two of the valid spots showed poor hybridization, the STDEV increased. Spots that were not considered valid were not included in the calculation of STDEV. For this study, the ideal standard deviation for each gene should be 0.3 or less. In general, the STDEV’s for the selected genes in Table 5.1 are less than 0.3. However, due to variation between each slide that occurred from user error and because two different hybridization temperatures were used, some of the genes showed an unfavorable STDEV (greater than 0.3). Thus, each gene (including genes with a higher than desirable STDEV) that showed initial promise as being an up- or downregulated gene was viewed and analyzed individually to ensure that the majority of valid spots showed a uniform fold change. Genes that did not present a majority of valid spots as being up- or downregulated were discarded from the list.

Analysis of the 10 nM PX time-course showed the number of genes upregulated increased significantly from the 3 to 8 h time-point. After the 8 h time-point, a small decrease in the number of genes upregulated was observed. Due to the low concentration of PX used, one would expect to see a sharp increase in the number of affected genes after the initial insult by PX followed by a decrease in changed gene number as the cell
moves back toward homeostasis. This trend was observed for upregulated genes over the 24 h time-course and is shown graphically in figure 5.1.

**Figure 5.1**

![Bar chart showing gene expression changes](image)

**Figure 5.1.** Significant changes in gene expression of SH-SY5Y cells following exposure to 10 nM PX. Genes listed show at least a 1.5 fold increase and -1.4 fold decrease compared to control. Total numbers of upregulated genes show a large increase from the 3 to 8 h time point with a modest recovery of normal phenotype by 24 h.
The number of down-regulated genes was similar in number at all time-points. The downregulated genes were also much smaller in number compared to upregulated, but this has been observed in other toxicology microarray results (Rogers et al. 2004). A few genes were affected at all three of the time-points. They included upregulated genes Cytochrome P450, family 2, subfamily C, polypeptide 8 (CYP2C8), Extracellular matrix protein 1 (ECM1), and Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1 (GNAI1) and the downregulated gene Ligase I, DNA, ATP-dependent (LIG1). A number of genes also showed significant fold changes in two of the three time-points. Three genes were upregulated in both the 3 and 8 h time points, while there were 5 genes that showed greater than 1.5 fold change in both the 8 and 24 h time points. Interestingly, using a cutoff of 1.4 fold upregulation increased the number of genes that showed upregulation in both 8 and 24 h from 5 genes to 18 genes. Even as the cell returned to stasis, PX was continuing to modulate a considerable number of genes.

While simple investigative conclusions can be made with the vast amount of microarray data, the question remains, what the hell does it all mean? Initial assumptions about the toxic effect of PX can be made by simply grouping the list of genes into general categories such as “cell cycle”, “structural”, or “transcription factors”. However, individual genes and their protein products have been shown to exhibit more than one molecular function as well as being involved in many biological processes within a cell. Classifying each gene into one category and trying to make hypotheses about what PX is doing in the cell can result in a shortsighted or incorrect view of the toxic effect.

The ability to turn an extensive amount of gene (or protein, etc) data into biologically relevant and understandable data has been addressed by a number of groups
The Gene Ontology (GO) project was initiated so as to develop a set of controlled and structured vocabularies to explain genes, gene products, and sequences for the science community (Ashburner et al. 2000; (GO) 2006). The ongoing project continues to develop and update the vocabulary as new scientific discoveries are made. "The GO project provides an ontological annotation system that enables biologists to infer knowledge from large amounts of data" ((GO) 2006). The systematic language used in describing each of the genes and/or gene products is comprised of information in three key areas shared by all organisms; molecular function, biological process and cellular component. The use of this functional information in combination with each of the genes showing differential transcriptional regulation can help emphasize the biological processes or pathways being modulated within an experiment. A number of programs have now been developed to help facilitate and streamline the interpretation of microarray data using the GO database. Included in this next section is an interpretation of the cDNA microarray data using the GoMiner software program.

**Functional analysis of microarray data**

GoMiner is a free program package that separates -omic data into biologically coherent categories using the GO database (Zeeberg et al. 2003). The user enters a list of all of the genes from the microarray slide along with a list of genes that were experimentally found to be changed in one way or another (up- or downregulated). The GoMiner output displays the total number of genes from the microarray slide associated with each of the specific GO categories including molecular functions, biological processes and cellular components. Underneath each of the category headings is the list of genes that were up- or down regulated that are associated with that category as
described by each gene’s ontology. Certain differentially-expressed genes can be located in numerous categories within molecular functions or biological processes. The GoMiner output also lists a number describing the relative enrichment (or depletion) of a certain category with respect to the marked genes as well as a two-sided $p$-value from Fisher’s exact test. The enrichment number starts at one (no enrichment or depletion) and moves up or down depending on the relative enrichment or depletion of the marked genes within each category. The $p$-value takes into account the number of total genes (from the user’s microarray slide) involved in each category and the number of marked genes in the exact same category that are either up- or down regulated from the experiment. The null hypothesis for each category’s $p$-value states- there is no difference between the proportion of flagged genes that fall into the category and the proportion of flagged genes that do not fall into the category. In other words, to reject the null hypothesis and observe a significant $p$-value means the number of flagged genes within a certain category is significant and not due to chance. A representative GoMiner output table is shown in figure 5.2.
Figure 5.2.

- **biological_process** (1036 1.03 p = 0.61 1.02 p = 0.31 1.02 p = 0.21)
- **cellular process** (993 0.93 p = 0.93 1.03 p = 0.28 1.01 p = 0.47)
- **cell adhesion** (100 0.44 p = 1.00 0.75 p = 0.81 0.62 p = 0.92)
- **cell communication** (437 1.14 p = 0.41 1.24 p = 0.05 1.23 p = 0.05)
- **cell-cell signaling** (99 1.14 p = 0.41 0.91 p = 0.66 1.01 p = 0.55)
  - CXCL13 (MGL,UniProt)
  - HMMR (MGI, RGO, UniProt, ZFIN)
- **transmission of nerve impulse** (42 1.10 p = 0.45 1.80 p = 0.14 1.78 p = 0.11)
- **regulation of action potential** (41 0.0 p = 0.00 3.11 p = 0.11)
- **synaptic transmission** (37 0.00 p = 1.00 2.04 p = 0.09 1.08 p = 0.17)
  - GRM1 (MGL, RGD, UniProt)
  - GRM3 (MGL, RGD, UniProt)
  - HTR2C (MGL, RGD, UniProt)
- **neurotransmitter receptor metabolism** (1 0.00 p = 1.00 15.08 p = 0.07 12.45 p = 0.06)
  - NRG1 (CGD, MGL, RGD, SGD, UniProt)
- **regulation of synapse structure and function** (8 0.00 p = 1.00 5.66 p = 0.01 4.67 p = 0.02)
- **regulation of synaptic transmission** (7 0.00 p = 1.00 6.46 p = 0.01 5.34 p = 0.01)
  - GRM3 (MGL, RGD, UniProt)
  - GNAI1 (MGL, RGD, UniProt, ZFIN)
- **negative regulation of synaptic transmission** (1 0.00 p = 1.00 15.08 p = 0.07 12.45 p = 0.08)
  - GNAI1 (MGL, RGD, UniProt, ZFIN)
- **regulation of synaptic plasticity** (5 0.00 p = 1.00 3.02 p = 0.29 2.49 p = 0.34)
  - GRIN1 (MGL, RGD, UniProt)
- **response to extracellular stimulus** (9 7 93 p = 0.12 1.69 p = 0.46 2.77 p = 0.16)
- **response to nutrient levels** (8 8 9.2 p = 1.11 1.89 p = 0.42 3.11 p = 0.13)
- **response to starvation** (8 8 9.2 p = 1.11 1.89 p = 0.42 3.11 p = 0.13)
  - **fruiting body formation** (sensu Dictyostelida) (4 17 85 p = 0.05 3.77 p = 0.24 6.23 p = 0.03)
- **signal transduction** (40 0.08 p = 0.77 1.31 p = 0.03 1.23 p = 0.05)
  - AKT3 (MGL, RGD, TAIR, UniProt)
  - CDK9 (DDB, FB, GeneDB, Spombe, MGL, UniProt, ZFIN)
  - CSF1R (MGL, RGD, UniProt, ZFIN)
  - CXCR4 (MGL, RGD, UniProt)
  - FCG2A (MGL, RGD, UniProt)
  - GNAI1 (MGL, RGD, UniProt, ZFIN)
  - GPR85 (MGL, UniProt)
  - GRIN3 (MGL, RGD, UniProt)
  - HTR2C (MGL, RGD, UniProt)
  - IL5RA (MGL, RGD, UniProt)
  - IQGAP1 (MGL, UniProt)
  - LAMC1 (FB, UniProt, ZFIN)
  - OXTR (MGL, RGD, UniProt)
  - PAK4 (MGL, UniProt)
  - PPP2R2B (RGD, UniProt, ZFIN)
  - PRRKAG1 (MGL, RGD, UniProt, ZFIN)
  - PRKAR1A (MGL, RGD, UniProt, ZFIN)
  - SOCS2 (MGL, RGD, UniProt)
  - SRF (DDB, MGL, UniProt, ZFIN)
  - STAT1 (MGL, RGD, UniProt, ZFIN)

**Figure 5.2.** A representative GoMiner output displaying GO categories under biological processes and corresponding up- and down regulated genes. The down
arrows (green) are downregulated genes, while the up (red) arrows are upregulated. The numbers before the p-value are the relative enrichments for the up- and down regulated genes.

Using the GoMiner software, the list of approximately 1600 genes from the Human Tox Microarray Chip were loaded into the program. In order to gain an overall insight into the action of PX on the cell, all of the up- and downregulated genes from the three time points were uploaded simultaneously. Also, because of the limited number of up- and downregulated genes in each time point, and the large number of GO categories, the use of all genes from all time points at one time would result in a more statistically significant assessment of altered biological processes. Data was obtained for the up- and downregulated genes in all three areas of GO, molecular functions, biological processes, and cellular components. The GO database defines molecular functions as any activity, such as binding, transcription, or receptor, at the molecular level. Biological processes are a series of events that include one or more molecular functions in order to achieve the biological process. The ensuing discussion will focus primarily on the biological processes in the cell that show differential gene transcription levels following PX insult.

Analysis of the GoMiner data resulted in eight biological processes showing induction of genes associated with those processes following PX exposure. These processes include cell communication, phosphorus metabolism, cellular proliferation, cellular lipid metabolism, cellular transport, cell differentiation, cell cycle, and cytoskeleton organization and biogenesis. The categories in bold are the broad category headings and are followed by a discussion of genes affected within the category and/ or
subcategories. An enrichment value of 0.0 and corresponding p value of 1.00 means no genes were up- or downregulated within that group.

Genes involved in cell communication (upregulated genes, relative enrichment 1.24, p=0.05, downregulated genes 1.14, p=0.41):

The largest group of upregulated genes was represented by cellular communication processes (437 total genes from Human Tox Chip involved in this process, relative enrichment of 1.24 for upregulated genes, p-value 0.05, relative enrichment of downregulated genes 1.14, p=0.41). This is a broad group that contains many subcategories and describes such pathways as cell-cell signaling and signal transduction. The signal transduction subcategory (404 total genes from Human Tox Chip) showed a p-value of 0.03 for upregulated genes and a relative enrichment of 1.31. Among the genes located in this category were Cyclin-dependent kinase 9 (CDK9), Colony stimulating factor 1 receptor (CSF1R), Chemokine receptor 4 (CXCR4), Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1 (GNAI1), G protein-coupled receptor 65 (GPR65), Interleukin 5 receptor, alpha (IL5RA), P21-activated kinase 4 (PAK4), and Protein phosphatase 2, regulatory subunit B, beta isoform (PPP2R2B). Sub-categories of signal transduction that contained upregulated genes included cell surface receptor linked signal transduction (relative enrichment of upregulated genes 1.39, p=0.08), G-protein coupled receptor protein signaling pathway (enrichment of upregulated genes 1.62, p=0.16), intracellular signaling cascade (enrichment of upregulated genes 1.32, p=0.12, enrichment of downregulated genes 1.47 p=0.28), and protein kinase cascade (up genes 1.45, p=0.18, down genes 1.72, p=0.33).
Signal transduction at the cellular level refers to the movement of signals from outside the cell to inside as well as intracellular signaling. The signal can be a simple movement of ions in and out of the cell through receptors or channels that can change electrical potentials and in turn propagate a signal through the cell (Alberts 2002). Signal transduction can also be a more complex pathway involving the coupling of ligand-receptor interactions to many intracellular events. These events include phosphorylations by tyrosine kinases and/or serine/threonine kinases. Protein phosphorylations play a critical role in signal transduction and can also change enzyme activities and protein conformations. The eventual outcome of signal transduction is an alteration in cellular activity and changes in the program of genes expressed within the responding cells. Phosphorylation is a critical regulator of signal transduction and the differential transcription of genes involved in signal transduction following OP exposure proposes an interesting hypothesis. PX might be interfering and/or disrupting different signaling cascades in the cell through unregulated phosphorylation of signaling cascade (i.e. protein kinase) targets.

Genes involved in phosphorus metabolism (upregulated genes, relative enrichment 1.49, p=0.04, downregulated genes 0.78, p=0.75):

The second major group of target genes included genes involved in the phosphorylation and dephosphorylation of proteins. Many of these genes generate protein products that are important in signal transduction. The ability of PX to interfere with the signal transduction pathways by phosphorylation correlates well with PX modulation of genes involved in the phosphorylation and dephosphorylation of signaling
cascades. Two subcategories of phosphorus metabolism contained nearly all of the changed genes. The first subcategory is protein amino acid dephosphorylation (upregulated genes 1.37, p=0.38) which included the genes Dual specificity phosphatase 6 (DUSP6), upregulated at the 8 h time point, and Dual specificity phosphatase 5 (DUSP5), which was upregulated at the 24 h time point. The second subcategory is protein amino acid phosphorylation (upregulated genes 1.48, p=0.08, downregulated genes 1.00, p=0.62) that was represented by a large number of upregulated genes involved in signal transduction. These genes included Protein kinase C (PRKC1), Protein kinase, cAMP-dependent, regulatory, type I, alpha (PRKAR1A) and Mitogen-activated protein kinase kinase 2 (MAP2K2), all upregulated at the 8 h time point and Mitogen-activated protein kinase kinase kinase kinase 2 (MAP4K2) which was upregulated at the 24 h time point. The inclusion of this biological process lends support to the idea that PX is phosphorylating targets in addition to AChE.

Genes involved in cell proliferation (upregulated genes 1.20, p=0.31, downregulated genes 0.00, p=1.00):

Cellular proliferation occurs by way of a complex collection of genes, proteins, and signaling cascades that are stimulated by any of numerous types of growth factors and stimulators. A considerable number of genes involved in cell proliferation were upregulated in response to PX throughout the 3, 8, and 24 h PX exposure time. They included Mannose-6-phosphate receptor (M6PR), Interleukin 5 receptor, alpha (IL5RA), and Low density lipoprotein-related protein 1 (LRP1). Further analysis of genes associated with cell proliferation noted both Ornithine decarboxylase (ODC1) and ECM1 as genes involved with proliferation but that were not included on the GoMiner list. The
majority of OP studies measuring cell proliferation after OP exposure show decreases in proliferation after a toxic dose (high concentration, micro- to millimolar) of OP (Mileson et al. 1998; Carlson et al. 2000; Guizzetti et al. 2005). However, studies have not been done correlating low dose OP exposure and cell proliferation. The hypothesis that low-level exposure to OP’s is implicated in cancer development (McDuffie 1994; Fritschi et al. 2005; Gwinn et al. 2005) could possibly be supported by the upregulation of genes in this group.

**Genes involved in cellular lipid metabolism (upregulated genes 1.57, p=0.21, downregulated genes 1.49, p=0.50):**

Members of this group were divided into two subcategories of cellular lipid metabolism, fatty acid metabolism (upregulated 2.26, p=0.14, downregulated 3.57, p=0.25) and lipid biosynthesis (upregulated 4.02, p=0.01). The majority of genes in this biological process were all upregulated during the 8 h time point. However, this group illustrates a possible problem in using the p values from GoMiner in determining whether or not there is a significant change to a certain biological process. The subcategory of lipid biosynthesis is made up of a possible 15 genes from the Human Tox Chip. Only two of these genes showed an upregulation, yet this resulted in a p value of 0.01. When a chip is designed that carries only a small number of genes within a certain category, a smaller number of up- or downregulated genes is needed to produce a significant p value. Caution should be used in interpreting biological classes seemingly affected by experimental treatment in relation to the types of genes that are contained on the microarray chip. This being said, the inhibition of carboxylesterases (esterases involved in cholesterol homeostasis and fatty acid metabolism, (Redinbo and Potter 2005)) by PX
might have secondarily caused the upregulation of these genes involved in cellular lipid metabolism.

**Genes involved in cellular transport (upregulated genes 1.36, p=0.07, downregulated genes 0.61, p=0.87):**

Cellular transport is another broad biological process that encompasses many different types of transport. Cellular transport can involve the movement of ions, electrons, chemical compounds, lipids, and proteins, within and in and out of the cell as well as genes involved in the regulation of cell transport. A significant number of genes were shown to be part of two different subcategories of cell transport. The first subcategory is intracellular protein transport (upregulated genes 1.46, p=0.22) and contained KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2 (KDEL2), that was upregulated in the 8 and 24 h time point, and Mannose-6-phosphate receptor (M6PR), a gene that is also shown to be involved in cellular proliferation. The second subcategory of cellular transport shown to have differentially transcribed genes was vesicle mediated transport (upregulated genes 2.74, p=0.01). The three genes listed by GoMiner for this biological process were SEC22 vesicle trafficking protein-like 3 (SEC22L3, 24 h), GDP dissociation inhibitor 2 (GDI2, 3 h), and Protein kinase C (PRKCI, 8 h). Vesicle mediated transport could be essential to cell survival through the removal of PX inhibited proteins or in the removal of PX alone.

The genes associated with **cell differentiation** (upregulated genes 1.23, p=0.25, downregulated genes 0.45, p=0.91), **cell cycle** (upregulated genes 1.08, p=0.43, downregulated genes 0.78, p=0.75), and **cytoskeleton organization and biogenesis**

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In this analysis we used Human Tox cDNA microarrays to analyze transcriptional changes during low-level, acute PX exposure in human SH-SY5Y neuroblastoma cells. Exposing the cells at three different time points (3, 8, 24 h) resulted in an increase of upregulated genes from the 3 to 8 h time point and a modest decrease of altered genes from the 8 h to 24 h time point. The number of downregulated genes was considerably less than upregulated genes and did not show a change in gene numbers throughout the time course. The majority of differentially altered genes (Table 5.1) showed minor but significant fold changes in the range of 1.5 and 2.0 for upregulated genes and -1.4 and -2.0 for downregulated genes.

Further analysis of genes was performed using the Gene Ontology database and the software program GoMiner. GoMiner compares the up- and downregulated genes from an experiment to the total genes from the microarray chip and groups the altered genes into biological processes or molecular functions depending on the unique ontologies of each gene. GoMiner produced eight different biological processes that PX might be modulating after exposure. These different processes involving up- and/or downregulated genes from the microarray experiments include cell communication, phosphorus metabolism, cellular proliferation, cellular lipid metabolism, cellular transport, cell differentiation, cell cycle, and cytoskeleton organization and biogenesis. A
considerable number of upregulated genes were grouped into cell communication, phosphorus metabolism, and cell proliferation.

Two recent studies have also investigated gene expression patterns following OP exposure using microarray. Changes in gene expression were measured in rats injected with sarin at an early time point of 15 min \((0.5 \times LD_{50})\) and a later time point of 3 months \((1 \times LD_{50})\), using a 1200 oligo gene chip geared toward functional and structural groups relevant to the nervous system (Damodaran et al. 2006). The early time point identified genes involved in ion channel, cytoskeletal, cell adhesion molecules, transporters, and G-protein coupled receptors. At three months, up- and downregulated genes were classified as calcium channel and binding proteins, cytoskeletal and cell adhesion molecules, and GABAergic signaling molecules. Differential gene expression has also been measured in human mammary epithelial cells exposed for 6 and 24 h to low concentrations \((0.2 \text{ mg/0.1 m}^3)\) of malathion (Gwinn et al. 2005). Genes showing differential expression after malathion treatment were found to be implicated in carcinogen and steroid metabolism, DNA replication, and cell cycle replication. These papers and the work presented in this chapter lend support and continue to build on the hypotheses that OPs target non-AChE-associated cellular pathways and further our understanding of the mechanisms of cellular toxicity.

Microarrays allow measurement and characterization of entire genomes and gene expression changes in a single experiment (Newton et al. 2004). However, due to the variability and possible error involved in microarray experiments, care must be taken in performing the experiment as well as confirmation of microarray gene changes by a secondary method. Possible sources of error include handling of RNA during isolation,
variable amounts of fluorescent probe added to the RNA, uniformity during the spotting
of the microarray chip (unequal amounts of cDNA), problems during hybridization
and/or washing (too much specific probe removed or too little non-specific probe
removed), and error in the data acquisition and analysis. Care was taken to minimize
error at each step, such as measurement of RNA after isolation (260/280 ratio), and
measurement of fluorescent labeling using the Nanodrop spectrophotometer. However,
the possibility of error still exists. Gene changes should be confirmed at the RNA and/or
the protein level. The confirmation of one-gene-at-a-time complements the discovery
role of the microarray methodology and ensures greater certainty in the microarray results
(Vrana et al. 2003). The work presented in the next chapter chronicles the experiments
done to confirm certain genes found to be differentially expressed by cDNA microarray.
Chapter 6

Confirmation of microarray data by semi-quantitative RT-PCR, western immunoblot and radioactive enzyme assay

Introduction

The power of microarray technology lies in the ability to monitor and characterize an extremely large number of genes and affords the possibility to identify novel genes involved in pathophysiological signaling pathways (Hoernli et al. 2004). The increased power of microarray is accompanied by amplified possibilities of errors and false positives. Increased attention to detail during microarray experiments as well as careful experimental design is imperative for successful results. However, due to the relatively early development of the technology and potential for false positives among the thousands of tested genes, confirmation of individual genes at the mRNA or protein level must accompany microarray data (Vrana et al. 2003).

Certain limitations are inherent in microarray experiments and are important to consider when analyzing the data (Vrana et al. 2003). First, microarrays measure relative amounts of RNA and not absolute amounts. Furthermore, cDNA microarrays are not very specific due to the length of the spotted target cDNAs (generally 500-2000 base pairs). This problem has been somewhat rectified with the use of oligos (25-50 bp) in place of cDNAs, however both cDNAs and oligos are not able to differentiate between alternatively spliced transcripts of the same gene or highly homologous members of the same gene family. Another challenge in using microarray is that certain assumptions are being made about the cellular targets, mechanisms and pathways being affected by a
certain treatment, yet the microarray is measuring an intermediate step, the mRNA levels, and not the levels of functional protein product. Once an mRNA is translated into a protein (if at all), many different post-translational modifications occur that regulates function of a protein. For these reasons and more, it is critical to experimentally confirm certain genes from the microarray data.

In this chapter, the cDNA microarray results will be attempted to be validated through confirmation of certain genes at the mRNA, protein, and enzyme activity level. These experiments will be carried out using semi-quantitative reverse transcription polymerase chain reaction (semi-quantitative RT-PCR) to make measurements at the RNA level, immunoblot to characterize genes at the protein level, and enzyme assays for analysis into whether transcriptional changes equate into differences in enzyme activity.

Results

Four specific genes were chosen to validate the microarray data. Two genes were upregulated (the specific reasons for choosing these two genes and further information about the genes will be explained more thoroughly in the following chapter), Ornithine decarboxylase 1 (ODC1, upregulated 2.4 fold, 8 h time point), and Extracellular matrix protein 1 (ECM1, upregulated 1.5, 2.2, and 1.5, at the 3, 8, & 24 h time points respectively); one gene was chosen as an internal control that showed no fold change at any of the time points in the microarray data, γ-Tubulin 1 (TUBG1); and the fourth gene was β-actin (BA), a standard housekeeping gene used as an internal control for the RT-PCR (Bustin 2000; Meadus 2003), that was not a target cDNA on the microarray chip.
Semi-quantitative RT-PCR

Individual gene mRNA levels were determined using semi-quantitative RT-PCR. The optimal annealing temperatures and the linear range for cycle number and quantity of template for each gene were determined empirically prior to comparing transcript amounts of PX and control treated mRNA. All genes characterized by RT-PCR were detected in the SH-SY5Y cells. Primers used in the PCR reactions are shown in Table 6.1. Following the RT-PCR reactions, PCR products were electrophoresed with ethidium bromide and optical densities of the resulting bands were measured and analyzed. Figure 6.1 shows the results of the semi-quantitative RT-PCR, comparing the optical density (OD) of the PX-treated transcript and the OD of the control-treated transcript.

Analysis of the RT-PCR data revealed no significant change between mRNA levels in PX-treated cells and mRNA in control-treated cells at any of the time points for any of the genes, compared to TUBG1 and β-actin controls. While elevated levels of PX transcript seem to be evident at the 8 h time point, they were also apparent in both the TUBG1 and β-actin controls, indicating an increased amount of total mRNA in the 8 h PX sample compared to the 8 h control mRNA.

Interestingly, an unidentified band appeared in all of the electrophoresed ECM1 PCR reactions. The expected bp size of the ECM1 transcript is 547 bp, yet in every reaction, another band was detected, approximately 100 bp above the expected band, creating a doublet. Gene sequence and primer analysis of ECM1 ruled out contamination with genomic DNA as well as the possibility of the band being one of the known splice variants. ECM1 is a relatively newly discovered gene, with the mouse gene first being

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cloned in 1994 and the human homologue isolated in 1997 (Chan 2004). There are currently three known splice variants of ECM1 (ECM1a, ECM1b, and ECM1c) showing differential expression (Fujimoto et al. 2005). This being said, the possibility of a novel splice variant that has yet to be characterized is reasonable. Calculations of the optical density of the ECM1 doublet showed differential expression compared to the four genes presented in Figure 6.1. Similar expression of mRNA levels between PX- and control-treated cells were seen at the 3 and 8 h time points, yet the 24 h time point showed an approximately 1.3 fold increase of PX over control, factoring in the TUBG1 and β-actin controls. The mRNA levels of the second unidentified ECM1 band are shown in figure 6.2. However, due to the scope of the project, further analysis of this uncharacterized doublet was not done.

**Immunoblot analysis**

To validate the mRNA changes characterized by the microarray results, SH-SY5Y cells were analyzed by immunoblot for changes in ECM1 and ODC1 protein levels following exposure to PX. Equal amounts of protein lysate were analyzed using an antibody to ECM1 that was provided by Professor Lurong Zhang at the University of Rochester, and a commercially available antibody against ODC1. After probing with the primary antibodies, membranes were stripped and re-probed for actin to ensure equal lane loading. Protein analysis was done with cells treated with 10 nM PX or acetone vehicle for 6, 12, 24 and 48 h. Optimal antibody and lysate concentrations were determined empirically prior to final experiments.
A significant increase in ECM1 protein level was observed at the 12 and 24 h time points (Figure 6.3). ECM1 showed upregulation of mRNA expression at the 3, 8, and 24 h time points in the microarray data. Protein expression of ECM1 was elevated at the 12 h time point and was maintained through the 24 h time point followed by a return to control levels by 48 h. Protein analysis of ODC1 was done in a similar fashion as ECM1. Results from the immunoblots showed no statistically significant changes in ODC1 protein levels from 6 h to 48 h (Figure 6.4)

**ODC1 enzyme activity assay**

ODC1 shows variable amounts of mRNA, protein and activity levels that do not always correlate within an experiment. Studies have shown minimal changes in mRNA, yet large inductions of protein level and even larger increases in enzyme activity (Lin et al. 2002). Other studies have observed changes in mRNA level and no changes in protein and activity (Klekner et al. 2001) while another study found all three to closely correlate (Mohan et al. 1999). For this reason, very small changes in mRNA as observed in the microarray data (but not yet confirmed by RT-PCR or immunoblot) might affect the activity of the enzyme. PX may induce a small upregulation of the gene (as seen by microarray) and/or is acting directly on the ODC1 protein to modulate enzyme activity. ODC1 enzyme activity was measured following PX exposure using a modified radioactive enzyme assay (Morgan 1997). Activity of ODC1 showed no statistically significant differences between PX and control treated cells (Figure 6.5). In general, *in-vitro* experiments measuring ODC1 enzyme activity after treatment with some type of compound use an average exposure time of 3 h. Measurements for this
experiment were done from 4 h up until an 8 h time point, the same time an increase in
mRNA was shown by microarray. However, it is believed modulated protein levels of
ODC1 are always accompanied by uniform changes in enzyme activity (Lin et al. 2002),
which is consistent with the data presented in this chapter.
Table 6.1 Primer sequences for semi-quantitative RT-PCR

<table>
<thead>
<tr>
<th>GENE</th>
<th>PRIMER SEQUENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODC1</td>
<td>5’-CTG CTT GAT ATT GGC GGT GGC TTT -3' (forward)</td>
</tr>
<tr>
<td></td>
<td>5’-TCA ACA ATC CGA TCG AGG CCA TCA -3' (reverse)</td>
</tr>
<tr>
<td></td>
<td>Expected product size = 413 bp</td>
</tr>
<tr>
<td>ECM1</td>
<td>5’-ATC TGC CTT CCT AAC CGT CAG CAT -3' (forward)</td>
</tr>
<tr>
<td></td>
<td>5’-AGG CCT TCC ATG TAC AGG TGT GAT -3' (reverse)</td>
</tr>
<tr>
<td></td>
<td>Expected product size = 547 bp</td>
</tr>
<tr>
<td>TUBG1</td>
<td>5’-TAG AGG GCT TTG TGC TGT GTC ACT -3' (forward)</td>
</tr>
<tr>
<td></td>
<td>5’-TGT TGA CCA TGG CGA TGT AGC AGT -3' (reverse)</td>
</tr>
<tr>
<td></td>
<td>Expected product size = 573 bp</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Expected product size = 303 bp</td>
</tr>
<tr>
<td></td>
<td>(Maxim Biotech, Inc., South San Francisco, CA)</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>5’-ATC AGT GTA AGG CAT GTG GCT CCT -3' (forward)</td>
</tr>
<tr>
<td></td>
<td>5’-AAG AGG AGC ATT GAG GAC CGT GTT -3' (reverse)</td>
</tr>
<tr>
<td>LRP1</td>
<td>5’-TAC ACA ATC TTC CGG TCT GTG CCA -3' (forward)</td>
</tr>
<tr>
<td></td>
<td>5’-TCA TCT ACT TTG CCG ACA CCA CCA -3' (reverse)</td>
</tr>
<tr>
<td>HMOX1</td>
<td>5’-CTT GAA CTT GGT GGC ACT GGC AAT -3' (forward)</td>
</tr>
<tr>
<td></td>
<td>5’-AGG AGA TTG AGC GCA ACA AGG AGA -3' (reverse)</td>
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Figure 6.1

Figure 6.1  Semi-quantitative RT-PCR of ODC1, ECM1, TUBG1, and B-actin genes at 3, 8, and 24 time points. Transcripts measured at each time point were done in triplicate using 10ng of template and 30 cycles of PCR. Graph is representative of two experiments, each performed in triplicate, carried out with different sets of cDNA.
Figure 6.2. mRNA levels of ECM1 unidentified 2nd band. Transcripts measured at each time point were done in triplicate using 10ng of template and 30 cycles of PCR. Graph is representative of two experiments performed with different sets of cDNA.
Figure 6.3. 

Immunoblot analysis of ECM1 following 10nM PX exposure. Optical densities (OD) were measured for each band and are graphically represented as (PX OD / actin OD) / (control OD / actin OD). Data is from three independent experiments. 12 and 24 h time points are statistically significant fold changes at the level of * p < 0.05 (unpaired t test).

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Figure 6.4

Immunoblot analysis of ODC1 following 10 nM PX exposure. Optical densities (OD) were measured for each band and are graphically represented as (PX OD / actin OD) / (control OD / actin OD). Each time point is representative of three independent experiments. There were no significant changes between any of the time points.
**Figure 6.5.** ODC1 enzyme activity following exposure to 10 nM PX. Activity of ODC1 was measured after 4, 5.5, and 8 h of PX exposure. Data represents experiments done in duplicate for each time point. The positive control was a 3 h media change prior to harvesting the cells (shown to strongly increase ODC1 activity, (Mattsson et al. 1984)).

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Discussion

The ability of microarray technology to characterize an immense number of gene expressions in one experiment increases the possibility of error within the experiment, namely the observation of false positives. For this reason, the validation of microarray data must be done by confirming gene changes by other accepted, well established techniques. This can include confirming changes from microarray data at the RNA and/or protein level. The validation of microarray data presented in Chapter 5 was addressed by three different methods which included semi-quantitative RT-PCR, western immunoblot, and a radioactive assay to measure enzyme activity.

The mRNA expression levels of four genes (ECM1, ODC1, TUBG1, and β-actin) were measured using semi-quantitative RT-PCR. The results from the experiments showed no change in expression levels in each of the three time points for TUBG1 (expected) as well as no change in expression levels of ECM1 and ODC1 (not expected, both genes showed upregulation in the microarray data). Further RT-PCR analysis measured PX and control treated cells mRNA expression of Cytochrome P450, family 2, subfamily C, polypeptide 8 (CYP2C8), Low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor) (LRP1), and Heme oxygenase (decycling) 1 (HMOX1). Again, no changes in expression levels between PX and control treated cells was observed. There could be any number of reasons the semi-quantitative results did not correlate with the microarray data. Because the gene fold changes in the microarray data were modest, the ability to observe the same modest changes through other methods becomes difficult and requires greater sensitivity and precision. The use of semi-
quantitative RT-PCR (sqRT-PCR) has drawn criticism as not being a sensitive enough method to monitor small changes in mRNA expression (Rajeevan et al. 2001). There is also the possibility that the cDNA microarray targets hybridized certain known and/or unknown splice variants, or gene family homologues, of these upregulated genes and these splice variants (or homologues) were not enriched using the chosen PCR primers. The increased expression of the unidentified 2\textsuperscript{nd} band of ECM1 at 24 h somewhat supports this hypothesis. Semi-quantitative RT-PCR can show variability in the results and is prone to error during the many steps from isolated RNA to electrophoresed PCR products (Brunner 2004). In some cases, the up- or downregulated genes from the microarray data are false positives and would therefore show no change by RT-PCR. The use of quantitative real-time RT-PCR has alleviated many of these problems and would have been a better choice for confirming microarray data. In addition to using semi-quantitative RT-PCR, upregulated genes ECM1 and ODC1 were also monitored for increased protein levels.

Immunoblot analysis of ECM1 and ODC1 confirmed the upregulation of the ECM1 gene and gave further proof to the possibility ODC1 was a false hit. ECM1 protein expression showed increased levels at 12 and 24 h post PX exposure. The microarray data shows a slight upregulation at 3 h (1.4 fold), followed by a substantial increase at 8 h (2.2 fold), and then a decrease at 24 h to 1.5 fold which correlates well with the protein expression. ODC1 protein expression did not change at any time point after PX exposures. Due to the variability in expression levels of ODC1 gene, protein, and enzyme activity discussed above, we wanted to make sure to analyze all possible
areas to either confirm or rule out ODC1 as a hit or false positive, and so ODC1 was further characterized with respect to enzyme activity.

The radioactive assay used to measure ODC1 activity showed no statistically significant increase or decrease in enzyme activity after PX insult. Initial failures in confirming ODC1 upregulation by semi-quantitative RT-PCR and western blot were attributed to two different factors. The first was that ODC1 is a highly regulated enzyme and has a very short half-life, ranging from minutes to an hour (Lin et al. 2002). The second reason is immortal cells express higher levels of ODC protein and exhibit elevated enzyme activity compared to normal tissue or primary cell lines (Hu et al. 2003). We believed the short half life in addition to high levels of ODC1 protein and activity (SH-SY5Y is an immortal cell line) was confounding our ability to measure small differences in either RNA or protein levels. However, after confirming no change in enzyme activity levels, we were confident in assuming ODC1 was a false positive (no change in RT-PCR, immunoblot, enzyme activity) and did not warrant consideration as an upregulated gene in response to PX. The work done on ODC1 emphasizes the importance in confirming up- and downregulated genes following microarray experiments.

While it is of utmost importance to confirm individual genes from the microarray data, the original hypothesis was to identify and characterize pathways, protein targets, or biological processes that are modulated in some way by the OP paraoxon. The next chapter specifies the reasons why ECM1 and ODC1 were chosen as genes to confirm and details the work done to support the hypothesis that 10 nM PX induces cellular proliferation.
Chapter 7

Characterization of the induction of cellular proliferation by acute, low-dose paraoxon exposure

Introduction

Microarray technology allows the user to simultaneously monitor thousands of gene expression levels in one experiment. The next step is organizing the extensive amounts of microarray data into intelligible biological data that sets forth different ideas of what all the gene changes mean. The interpretation of this data is often the most difficult step of a microarray experiment.

There are a number of programs available that analyze the different up- and downregulated genes and attempt to produce different pathways or biological processes that might be modulated in some way. While the organization of the microarray data into the different pathways is extremely helpful, it does not establish proof of causation. Nevertheless, the organization of this data is advantageous in developing hypotheses about the nature of the treatment and its effect on a biological or cellular system. The next step after carefully reviewing and evaluating the meaningful microarray data is to design an appropriate set of experiments to test a hypothesis.

The purpose of the present research was to test the hypothesis that the OP PX modulated / altered / blocked a unique or novel set of protein targets or specific cellular pathways following exposure. Microarray data from SH-SY5Y cells treated with 10nM PX was further characterized using the GoMiner software to try and identify these affected targets or pathways. The genomic changes identified by microarray generated eight possible affected cellular processes organized and elucidated by GoMiner. These
processes included signal transduction, phosphorus metabolism and cellular proliferation. With the preponderance of experimental data showing OPs decrease or inhibit cellular proliferation at micromolar to millimolar concentrations (Rodriguez and Bustos-Obregon 2000; Guizzetti et al. 2005), we hypothesized that a low concentration (nanomolar) dose of PX might actually stimulate proliferation.

After deciding to further investigate the effect of PX exposure on cellular proliferation, a more careful analysis was performed on the up- and downregulated genes to identity any other genes identified in the microarray experiment that might be involved in cellular proliferation, but were not identified by GoMiner. Two other genes, in addition to the genes named by GoMiner, Ornithine decarboxylase 1 (ODC1) and Extracellular matrix protein 1 (ECM1) were classified as genes involved in cellular proliferation. These were also the two genes that chosen for the validation of the microarray results (previous chapter). In addition, both ODC1 and ECM1 were very good hits from the microarray data, with a high number of valid spots, low STDEV, and a significant fold change.

ODC1 is a key, rate-limiting enzyme in the biosynthesis of polyamines in mammalian cells (Shantz and Pegg 1999; Zhang and Chen 2002; Hu et al. 2003). The polyamines putrescine, spermidine, and spermine are essential for cellular growth, differentiation and proliferation (Mohan et al. 1999). Upregulation of ODC1 gene, protein level, and enzyme activity has been implicated in the induction of cell proliferation, and ODC1 has also been shown to be constitutively expressed at increased levels in cancer (immortal) cells (Mohan et al. 1999; Klekner et al. 2001; Hu et al. 2003).
The choice of ECM1 as a gene to use to both confirm the microarray data and characterize its existence in SH-SY5Y cells, is due to its role in proliferation as well as ECM1 being a relatively newly discovered protein. ECM1 is a widely expressed glycoprotein that has been characterized in cell proliferation, angiogenesis, differentiation, as well as regulation of basement membrane and growth factor binding within the skin (Wang et al. 2003; Chan 2004; Lupo et al. 2005). ECM1 consists of three known splice variants ECM1a, ECM1b, and ECM1c. ECM1a is widely expressed and is found in various tissues including skin, liver, lung, ovary, prostate, skeletal muscle and heart. ECM1b on the other hand has only been discovered in tonsils, keratinocytes, and tissue of the upper respiratory tract (Fujimoto et al. 2005). ECM1c has yet to be fully characterized with respect to its distribution and expression, but has been shown to account for approximately 15% of skin ECM1 RNA (Chan 2004). Protein levels of ECM1 have shown increased expression in malignant epithelial tumors (breast and lung) (Han et al. 2001; Wang et al. 2003) and have has been used as a diagnostic marker in malignant thyroid neoplasms (Kebebew et al. 2005).

Because a considerable number of genes (ten) involved in cellular proliferation showed an upregulation in response to 10 nM PX exposure, experiments were devised to test the hypothesis that PX causes an increase in proliferation. The data presented in this chapter documents the experimental work done to prove the idea that exposure to 10 nM PX induces cellular proliferation.
Results

In order to assess the potential effect of 10 nM PX on cellular proliferation, growing SH-SY5Y cells were used. SH-SY5Y cells have been used previously as in vitro models to study and characterize neuronal growth (Matthews and Feldman 1996; Celli et al. 1999). Two different experimental approaches were taken to identify cellular proliferation; manual cell counting and MTT assay.

The manual cell counting method involved plating cells in 6 well plates and 48 h later treating with 10 nM PX or acetone control. After 6 days of PX treatment, cells were removed from the plate and counted (Figure 7.1). Cells treated with 10 nM PX showed a significant (p<0.005) increase in cell number compared to cells treated with acetone vehicle. In general, about a 1.3 – 1.4 fold increase in cell number was observed throughout the replicate experiments. To rule out the possibility that PX was interacting with a component in the medium, which in turn secondarily caused cell proliferation, media was pre-treated with 10 nM PX and allowed to incubate at 37°C for 48 h before addition to the cells. Pre-treatment of media with PX resulted in no significant (p>0.05) proliferation relative to control (Figure 7.2).

To supplement and confirm the cell counting experiment, cellular proliferation in SH-SY5Y cells was also assessed by MTT assay. The MTT assay is a colorimetric assay that analyzes proliferation by measuring the cleavage product (purple formazan crystals) of yellow tetrazolium salt MTT by the mitochondrial enzyme succinic dehydrogenase, which is active in proliferating cells. SH-SY5Y cells were treated with 10 nM, 1 nM, or acetone vehicle for 4 days and then analyzed for cellular proliferation using the MTT
assay. Results are shown in Figure 7.3. The MTT assay showed an approximate increase in cellular proliferation of 1.2 fold following 10 nM PX exposure. Treatment of SH-SY5Y cells with 1 nM PX showed no increase in proliferation compared to control.
**Figure 7.1. 10 nM PX induces cellular proliferation.** SH-SY5Y cells were passed into a 6 well plate at 1x10^5 cells/well. Treatment started 48 h post plating. Cells were counted 6 days after treatment using a hemacytometer. Graph representative of three experiments, each done in triplicate. **p<0.005, (unpaired t test)**
Figure 7.2. Media pre-treated with 10 nM PX does not induce cellular proliferation. SH-SY5Y cells were passed into a 6 well plate at $1 \times 10^5$ cells/well. Media was treated with 10 nM PX 48 h and allowed to incubate at 37°C before addition to the cells. Cells were counted 6 days after treatment using a hemacytometer. Graph representative of two experiments, each done in triplicate. $P>0.05$ (unpaired t test)
Figure 7.3. Measurement of SH-SY5Y cellular proliferation by MTT assay. SH-SY5Y cells were passed into a 96 well plate at 1 and 3x10^4 cells/well. Treatment started 48 h post plating. Cells were measured for proliferation 4 days after treatment using an MTT assay. Graph representative of six experiments (three at 1x10^4 and three at 3x10^4 cells/well), each done in triplicate. Similar changes were observed for 1 and 3x10^4 cells/well. * p<0.05, (unpaired t test)
Discussion

Results from the cell counting experiment and MTT assay corroborate our hypothesis that low dose (10 nM) PX induces cellular proliferation. Two approaches were used to evaluate cellular proliferation induced by PX. In the first, human neuroblastoma SH-SY5Y cells showed increased proliferation of about 1.3-1.4 fold following a 6 day treatment of 10 nM PX. The cells also showed a significant (p<0.05) increase of 1.2 fold by MTT assay after 4 days of 10 nM PX exposure. However, the increase in proliferation was reduced when the concentration of PX was ten fold less (1 nM). The MTT assay (4 day PX treatment) might have shown a similar fold change to the cell counting experiment (6 day PX treatment) if the PX exposure was carried out an additional two days. However, the small quantity of media in the 96 well plate did not allow for this.

The MTT assay measures the activity of a mitochondrial enzyme, so it is appropriate to ask whether OPs directly target mitochondria. Research has shown that exceedingly high levels (100 μM – 1 mM) of OPs hyperpolarize the mitochondrial transmembrane potential in SH-SY5Y cells (Carlson and Ehrich 1999). However, the change in potential was not directly correlated to OPs and is possibly the secondary result of OP cytotoxicity (apoptosis).

A number of other cell counting experiments were performed to further characterize cellular proliferation. Media was pre-treated with 10 nM PX and allowed to incubate at 37°C for 48 h prior to addition to the cells, which resulted in no increase in cellular proliferation. This experiment ruled out the possibility that PX was reacting with
components in the media which secondarily caused cellular proliferation. Multiple doses of PX throughout the 6 day treatment period were also experimentally examined. The results of this experiment again showed no increase in cellular proliferation compared to control. This might be due to the fact that each successive dose resulted in increased concentrations of PX, therefore blocking the proliferation phenotype.

There are different methods to measure cellular proliferation in addition to cell counting by hemacytometer and MTT assay. Using the same experimental setup as the cell counting by hemacytometer, SH-SY5Y cells were also counted using a Coulter Counter. Results from the Coulter Counter were quite varied. In one experiment there would be induction of proliferation followed by an experiment that showed no induction or even a decrease in cellular proliferation. The tendency of SH-SY5Y cells to aggregate and form clumps resulted in inconsistent counts with the Coulter Counter. A research group measuring cellular proliferation in SH-SY5Y cells noted that the MTT assay is the superior choice for measuring proliferation in this specific cell line (Celli et al. 1999).

A comprehensive literature search uncovered a single paper that investigated low level OP exposure and cell proliferation. The OP methamidophos stimulated cell proliferation of SH-SY5Y by 28% at a concentration of 700 nM, and inhibited proliferation by 62% after exposure to 7 mM as determined by MTT assay (Li et al. 2003). Calcium uptake was also measured following exposure to methamidophos. SH-SY5Y cells showed decrease uptake and markedly increased uptake of calcium at high and low levels of methamidophos, respectively. Their conclusion was that methamidophos was disrupting calcium homeostasis which was leading to the increase or inhibition of proliferation depending on the concentration of OP. While the work
discussed in this chapter shows a similar end result, the data presented here provides
evidence that 10 nM PX is upregulating a number of cellular proliferation genes resulting
in the induction of proliferation.

Interestingly, a number of studies have linked occupational exposure of OP
pesticides with an increased risk of non-Hodgkin’s lymphoma (McDuffie 1994; Fritschi
et al. 2005). Another study demonstrated that malathion and parathion (0.167xLD$_{50}$ and
0.5x LD$_{50}$, respectively, 2 times daily for 5 days) induced proliferation of terminal end
buds of mammary glands followed by formation of mammary carcinomas in Sprague-
Dawley female rats (Cabello et al. 2001). OPs, as a general class, have not been
characterized as carcinogens, but these studies and the fact that 10 nM PX induces
cellular proliferation support the hypothesis that OPs may be implicated in cancer.
However, caution should be taken in interpreting the $in$ vitro data from this chapter in that
it does not take into account the toxicokinetic factors of an $in$ vivo model.

The cell cycle is a highly regulated process that is tightly associated with cellular
proliferation. There a large variety of signals, including growth factors, cytokines, and
mitogens, that induce cells to divide (Alberts 2002). The signals that induce proliferation
are moved through a number of different signaling pathways that include the MAP
kinase, Protein kinase C, and JAK/STAT pathways (Alberts 2002). Further work would
need to be done to identify if the upregulation of cellular proliferation genes, and the
increased levels of ECM1 protein, are due to direct action of PX acting as a growth
factor, or are a secondary result of a target or signaling pathway being modulated by PX,
which in turn leads to an upregulation of proliferation genes. Also, because this work
was done in an immortal cell line, which already have a number of proliferation genes
constitutively expressed, additional experiments would need to be done on primary cell lines to see if low dose PX induces the same phenotype.
The initial discovery and development of OPs led to the characterization of the main mechanism of OP toxicity, the inhibition of AChE. Further work defined the consequences of this inhibition, namely cholinergic crisis. Recent research into OPs toxicity has identified other significant secondary targets and outcomes of OP exposure that include OPIDN and OPICN. While the clinical manifestations of OP exposure, outside of AChE inhibition, have been fairly well characterized, the cellular components, mechanisms and pathways that are involved in the disease's progressions are obscure. The work presented in this thesis begins to characterize the possible pathways and targets altered by OP exposure. Using cDNA microarray, transcriptional changes in the SH-SY5Y human neuroblastoma cell line were measured after treatment with 10 nM paraoxon (PX). The results of this experiment led to the following conclusion:

- **Acute, low-level exposure to PX induced the up- and downregulation of a number of genes.** Using GoMiner software, it was determined that a number of different cellular processes were modulated following PX exposure. Analysis of the GoMiner data resulted in eight biological processes showing induction of genes associated with those processes. These processes include, cell communication, phosphorus metabolism, cellular proliferation, cellular lipid metabolism, cellular transport, cell differentiation, cell cycle, and cytoskeleton organization and biogenesis.
Because OPs have been implicated with the development of cancer and the fact that a number of genes from the microarray data were grouped under cellular proliferation, experiments were devised to test the hypothesis that exposure to 10 nM PX induces cellular proliferation. The findings of these experiments are summarized below:

- **10 nM PX exposure induced significant cellular proliferation in SH-SY5Y human neuroblastoma cells.** Using two different techniques to measure cellular proliferation, it was shown that exposure to 10 nM PX induces a small, but significant proliferation. The MTT assay showed about a 1.2 fold increase in proliferation at 10 nM PX while 1 nM PX showed no increase. The cell counting experiment measured an approximately 1.3 fold induction of cellular proliferation. Further work needs to be done to fully characterize changes in proliferation at different time points and different concentrations of PX.

The microarray data in chapter 5 was attempted to be confirmed using a number of different techniques including semi-quantitative RT-PCR, immunoblot, and enzyme activity assay. A unique finding from these confirmation experiments is detailed below:

- **Expression of ECM1 in SH-SY5Y human neuroblastomas was confirmed by RT-PCR and immunoblot.** ECM1 is a recently discovered protein that has yet to be fully characterized with respect to its expression. ECM1 expression has been identified in a number of tissues including skin, blood vessels, heart, placenta, tonsils, liver and lung.
By northern blot, ECM1 expression was not detected in brain, but neuroblastomas have not been tested for ECM1 expression. The characterization of ECM1 by RT-PCR and immunoblot in SH-SY5Y confirms the presence of the gene and protein. Exposure to 10 nM PX induces the increased expression of protein levels as shown by microarray and immunoblot.

Overall, the work detailed in this study contributes to an initial understanding of gene changes in response to PX exposure. The microarray data presented here resulted from the use of a Human Tox cDNA chip. The use of a full genome oligo microarray chip would allow the researcher the opportunity to more fully characterize genomic changes after PX exposure. Additional experiments with different concentrations of PX, or different types of OPs, and different time points would also be advantageous in creating toxic footprints of all types of OPs. Examination of data from different OPs and concentrations might also identify possible biomarkers of OP exposure. OPs as a class might upregulate certain genes they all have in common, but also a characteristically unique set of genes by which to identify specific OPs. The identification of biomarkers would be useful in developing screening techniques for OP exposure in humans. Following the additional microarray work, experiments devised to test specific pathways and protein targets could lead to a better understanding of OPs toxicity and clinical outcomes. Clearly, further work is needed in characterizing the protein targets and cellular pathways affected by OPs.

The identification of specific cellular machinery involved with proliferation following OP exposure is also important. From the work presented here, it is not possible
to determine whether PX is the primary cause of proliferation or if proliferation is a secondary result of some other PX interaction within the cell. The examination of the ability of different OPs to cause proliferation, and the exploration into whether or not OPs cause proliferation in other cell lines and specific tissues would also need to be performed.
REFERENCES


