DISSERTATION

AN INTEGRATIVE STUDY FOR DEVELOPMENTAL NEUROTOXICITIES OF METHYLMERCURY AND PCBS: COMBINATION OF COMPUTATIONAL MODELING WITH EXPERIMENTAL TOXICOLOGY

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SUN KU LEE ENTITITLED AN INTEGRATIVE STUDY FOR DEVELOPMENTAL NEUROTOXICITIES OF METHYLMERCURY AND PCBS: COMBINATION OF COMPUTATIONAL MODELING WITH EXPERIMENTAL TOXICOLOGY BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

AN INTEGRATIVE STUDY FOR DEVELOPMENTAL NEUROTOXICITIES OF METHYLMERCURY AND PCBS: COMBINATION OF COMPUTATIONAL MODELING WITH EXPERIMENTAL TOXICOLOGY

The underlying purpose of the study is to investigate the potential whether MeHg and PCBs can induce interactive effects in the developing brain using experimental toxicology and computational modeling. To assess interactive effects between MeHg and PCBs in mice, we focused on two different perspectives: pharmacokinetic interactions and pharmacodynamic interactions. For the former studies, we first investigated pharmacokinetic interactions between two representative PCB congeners. Co-administration of PCB 153 and PCB 126 increased PCB 153 retention in the liver and decreased PCB 153 accumulation in the fat of non-pregnant mice. However, little or no significant pharmacokinetic interactions were observed in lactating mice and suckling pups. To describe pharmacokinetic interactions between PCB 153 and PCB 126, a physiologically-based pharmacokinetic model for PCB 153 disposition was developed. The effects of PCB 126 on the fat content in liver and a diffusion permeation constant in fat were incorporated into the PBPK model. The refined PBPK model adequately described pharmacokinetic interactions. Another PBPK model was constructed to describe the mass transfer of PCB153 into the developing pup during lactation by incorporating the changes in the volume and blood flow into mammary tissues, and additional mechanistic changes. Then, we investigated pharmacokinetic interactions

between MeHg and PCB congeners. The experimental results showed that co-exposure with PCB congeners increased the lactational transfer of MeHg to the pups and compensated the plasma levels of albumin, which decreased by the exposure of MeHg only. A refined PBPK model quantitatively described the pharmacokinetic changes of MeHg by co-exposure with PCBs in both maternal and pup's tissues and suggested the possible mechanism of pharmacokinetic interactions. For the pharmacodynamic studies, we analyzed protein expression profiles of mural pups exposed to MeHg and/or PCB congeners in cerebellum and hippocampus using proteomics and western blot techniques. The affected proteins were diverse including structural, glycolysis-related, Ca^{++}/calmodulin signal transduction-related, energy balance-related, growth related and stress-related proteins. The expression patterns of proteins were different between single chemical treatment and chemical mixture treatment. Our approach combined experimental toxicology and computational modeling and will ultimately contribute to the innovations of risk assessment in developmental neurotoxicology.

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List of Abbreviations

AAALAC	Association for assessment and accreditation of laboratory animal care international
ACTH	Adrenocorticotrophic hormone
Ah	Aryl hydrocarbon
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BCA	Bicinchoninic acid
BCG	Bromocresol green
BDNF	Brain-derived neurotrophic factor
CAK	Cyclin-dependent kinase-ctivating kinase
CaMK	Calcium/calmodulin kinase
ChAT	Choline acetyl transferase
CID	Collision-induced dissociation
CNS	Central nervous system
CREB	Cyclic AMP-response element binding protein
CYP	Cytochrome P450
DTT	Dithiothreitol
EPA	Environmental protection agency
ESI	Electrospray ionization
GABA	Gamma aminobutyric acid
GD	Gestational day
GFAP	Glial fibrillary acidic protein
GFR	Glomerular filtration rate
GRF	GDP releasing factor
GST	Glutathione S-transferase
HBSS	Hank's balanced salt solution
hCG	human chorionic gonadotrophin
hPL	human placental lactogen
HPLC	High pressure liquid chromatography
HSP	Heat shock protein
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LTD	Long term depression
LTP	Long term potentiation
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MAPK	Mitogen-activated protein kinase
MeHg	Methylmercury
MSH	Melanocyte stimulating hormone
NGF	Nerve growth factor
NMDA	N-methyl-D- aspartate
NT	Neurotrophin
PBPK	Physiologically-based pharmacokinetic
PBPK/PD	Physiologically-based pharamockinetic/pharmacodynamic

PCB	Polychlorinated biphenyl
PCB 74	2,4,4',5-tetrachlorobiphenyl
PCB 126	3,3',4,4',5-chlorinated biphenyl
PCB 153	2,2',4,4',5,5'-chlorinated biphenyl
pI	Isoelectric point
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PND	Postnatal day
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodesyl sulfate-polyacrylamide gel electrophoresis
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TGF	Transforming growth factor
TFA	Trifluoroacetic acid
Trk	Tropomyosin receptor kinase
UDPGT	UDP-glucuronyltransferase
VLDL	Very low-density lipoprotein

CHAPTER 1

Introduction: Review of the Literature and Significance of the Research

Sun Ku Lee

This dissertation consists of several areas such as neurodevelopmental toxicology, perinatal pharmacokinetics and pharmacodynamics, physiologically-based pharmacokinetic (PBPK) modeling, and proteomics in the developing brain. All of these areas were studied in mice following exposure to methylmercury (MeHg) and PCB congeners (i.e., PCB 153 and PCB 126) singly or in combination as chemical mixtures. Therefore, review of the literature described below was categorized with special emphasis on the following seven areas: (1) Developmental Neurobiology; (2) Developmental Neurotoxicology; (3) Perinatal Transfer of Chemicals; (4) Toxicology of Chemical Mixtures; (5) Developmental Neurotoxicology of MeHg and PCBs; (6) Biologically-based Computational Modeling: PBPK Modeling and Boolean-Network Modeling; and (7) Proteomics. The purpose of this review is to introduce a preliminary understanding of each individual area.

I. Developmental Neurobiology

A. Overview of Brain Development

During early development, the embryo is converted into a three-layer structure: ectoderm, endoderm, and mesoderm. This process is known as gastrulation.

Vertebrate nervous system is derived from the ectoderm (Gilbert, 2000). After gastrulation, the neural tube and the neural crest form from the ectoderm. The central nervous system (CNS) is derived from the neural tube and peripheral nervous system is derived from the neural crest (Gilbert, 2000). The neural tube is initially a thin-walled structure with a large central lumen, or neural canal. The walls consist of pseudostratified neuroepithelium, which proliferates rapidly and differentiates into the precursor cells that will eventually constitute the CNS (Stein *et al.,* 2002). Neurons develop from precursor cells that migrate into the intermediate zone. The migration of those cells is assisted by glial cells. Migratory process propels cells through microenvironments determining patterns of gene expression, the cell differentiation, and the timing of maturation. The pattern of cell proliferation produces a shallow groove along the inner lateral surface of the walls on each side (Stein *et al.,* 2002). This groove extends the entire length of the future spinal cord and as far as the future midbrain. The developing neural tube is surrounded by primitive mesoderm that differentiates into connective tissue.

From its inception at neural induction, the nervous system is organized along two axes: the anteroposterior axis and mediolateral axis (Gilbert, 2000). These two axes coordinate with each other to form specific neuronal patterns. The axes are first established according to the positional information produced by the maternal morphogen bicoid (Squire, 2003). The bicoid gradient initiates a cascade of transcription factors, which progressively subdivide the body. Following the establishment of polarity, subsequent patterning events subdivide the neural tube into smaller organizational units. Intrinsic and extrinsic signaling systems refine

positioning in a subtle way (Squire, 2003). The following picture shows the brief description of early brain development.

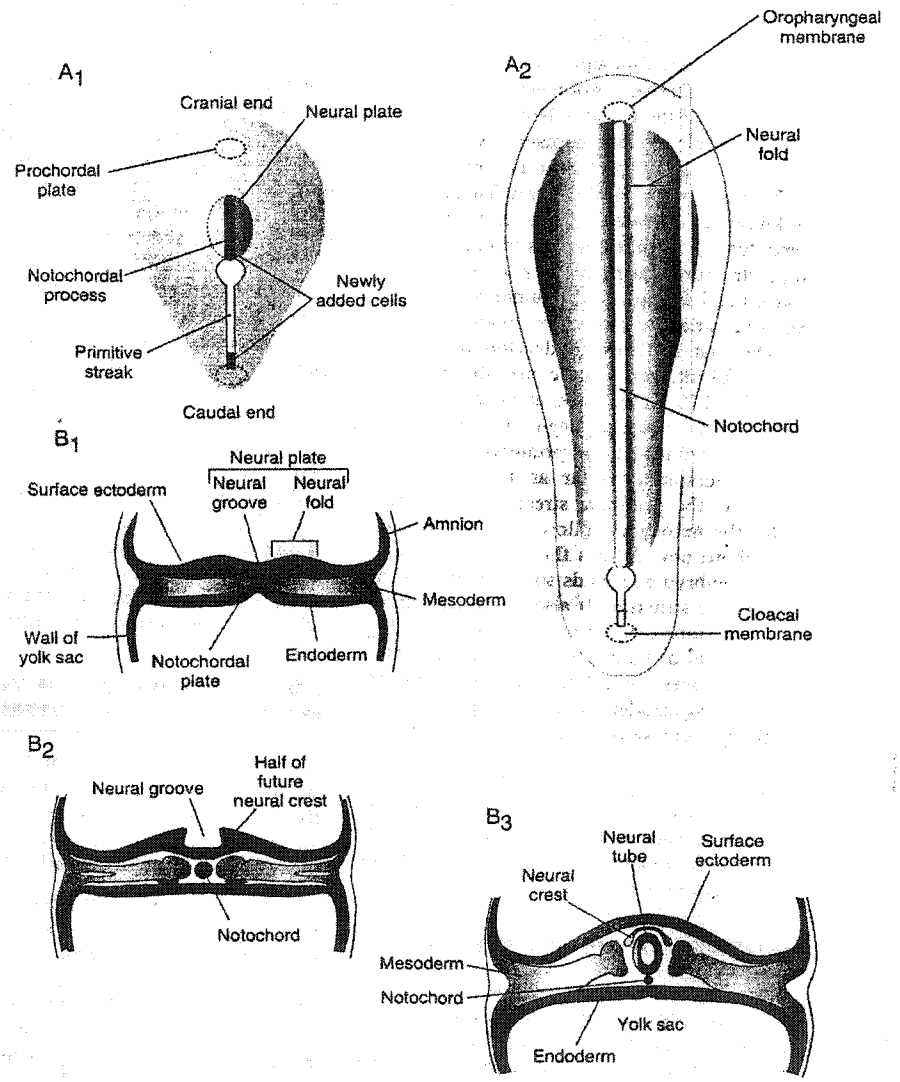

Fig. 1.1. Early brain development in vertebrates. A1: Formation of the neural plate. A2: Extension of the neural plate. B1: Division of the neural plate. B2: Formation of the neural tube. B3: Formation of the neural tube and the neural crest.

B. Fate Determination of Neural Cells

During neurodevelopment, neural cells express many different anatomical, physiological, and biochemical characteristics. Some of these characteristics are transient; others are permanent (Gilbert, 2000). The distribution of neurons with particular characteristics shows a unique pattern in each species. In general, two different factors determine the fate of neural cells: extrinsic cues, and intrinsic cues. Extrinsic cues are the components in the local environments where neurons proliferate and differentiate. They are composed of diffusible molecules, cell-membrane attached factors, and extracellular signaling molecules (Squire, 2003). Extrinsic cues act via membrane receptors on the responding cell to initiate signal transduction cascades and alter the expression of genes important for cell fate. Intrinsic cues are composed of intracellular molecules and proteins inherited from the precursor neurons (Squire, 2003). Generally, intrinsic factors exert their actions via intracellular signal transduction pathways. The production of diverse neuronal and glial phenotypes is accomplished by an elaborate interplay between intrinsic and extrinsic cues.

C. Pathfinding and Synapse Formation

Developing neural cells need to be localized to their appropriate sites in the brain. This process is called as the pathfinding. Growing neurons (i.e., growth cones) crawl forward in order to find their appropriate targets. Guidance cues are likely to direct growth cones through altering the location or rate of actin polymerization. The leading edge of a growth cone adheres to a permissive surface,

resulting in actin polymerization. At the same time, actin depolymerization occurs at the rear side. These processes will move the growth cones to the target site (Squire, 2003). Once in the target field, growth cones may arborize widely so that the correct connections will be expanded and incorrect connections will be retracted (Spector, 1965).

The arrival of growth cones at target sites initiates neurochemical and morphological events creating synapses. The establishment of a synapse following the initial contact between a growing axon and its target cell involves an exchange of signals including the differentiation of its partner. Growing neurons synthesize and secrete agrin into the extracellular matrix at synaptic sites. Agrin triggers to cluster acethylcholine receptors at the synapse and initiates postsynaptic differentiation (Rudolf, 1992).

D. Apoptosis in Neurodevelopment

During the entire developmental period, all neurons and glial cells undergo apoptosis. The survival of developing neurons depends on a variety of signals, involving multiple neurotrophic factors playing roles in maintaining survival and regulating differentiation. Neurons compete for limiting amounts of neurotrophic factors. If growing neurons cannot receive signals from neurotrophic factors, these neurons will die and disappear by apoptosis. Through this process, apoptosis may contribute to establishing optimal levels of neuronal populations in the developing nervous system (Margolis *et al.,* 1994).

Neurotrophic factors play critical roles in preventing neuronal death, maintaining neuronal populations, and inducing plasticity (Squire, 2003). In general,

neurons require neurotrophic factors for survival. Most neurons require multiple neurotrophic factors derived from multiple families. Some neurons normally switch their neurotrophic dependencies from one factor to another during development. In addition, some neurotrophic factors may serve as repair molecules available for a rapid response to injury in the mature nervous system (Rudolf, 1992). The receptors of neurotrophic factors in neurons are similar to the receptors of growth factors in other tissues. Many neurons have both high- and low- affinity binding sites for neurotrophic factors. Biological responses are associated with high-affinity binding and rapid phosphorylation. Binding of neurotrophic factors with their receptors initiates receptor dimerization and phosphorylation of cytoplasmic tyrosine residues. The phosphotyrosines recruit cytosolic and membrane-associated proteins involving the activation of growth factors and related signal transduction pathways (Squire, 2003).

E. Functional Development during Critical Period

Although synaptic connections in the nervous system are capable of adaptation throughout the lifetime of the organism, many connections begin to form firmly at specifc periods during the later stages of their development. The capacity for adjustment is substantially greater in developing brain than in the adult brain. This period is called the critical period. During the critical period, neural connections wait for the specific information, encoded by impulse activity in order to develop normally. This information causes the connection to commit irreversibly among many possible connections. If appropriate information is not obtained during the critical period, the

connection never has the ability for processing neurological information and neuronal functions are permanently impaired (Squire, 2003).

Three theories of functional brain development have been proposed (Purves, 2001). First, cognitive abilities develop depending on the maturation of the cortical area. Second, cognitive abilities develop as the networks of cortical areas form the appropriate interactions. Third, cognitive activities become active after receiving optimal inputs during the critical period. In addition to the brain maturation, functional brain development depends on experience and neural activity. The interaction with environments can also help to form inter- and intra-regional connections within the cortex.

II. Developmental Neurotoxicology

A. Significances and Limitations in Developmental Neurotoxicology

An important perspective of developmental neurotoxicology is that certain toxicants may induce more severe damages to the nervous system during development than at maturity. Toxicants can affect normal neurodevelopment by diverse mechanisms. For example, some toxicants can induce significant deficits in the brain directly during developmental exposure. The other toxicants can augment the extent of severity on neurologic diseases or change normal developmental signals in wrong directions.

There are some limitations in exploring developmental neurotoxicology. For example, our understanding on the relationships between morphological development and functional development is limited. Sometimes even though the brain is morphologically normal, neurologic functions may be affected (Hollander, 1997).

Besides, the development of the nervous system is complex in many ways. Each neuron has a unique integrative capacity derived from its individual morphology, neurochemical properties, and connections. Neuronal diversity is dependent upon cellular interactions during development. During a short time period, the developing brain expands dramatically and increases cellular heterogeneity over hours, days, and weeks (Squire, 2003). Epidemiological studies show that environmental toxicants induce developmental neurobehavioral impairments (Gilbert, 2000). These findings are consistent with the suggestion that interactions between genetic and environmental factors are critical determinants in developmental neurodeficits.

B. Mechanistic Considerations on Developmental Neurotoxicology

In general, developmental neurotoxicants can be identified by the detection of morphologic or functional alterations at various times after exposure during development. The mechanisms of developmental neurotoxicants can be characterized by their effects on the developmental events such as proliferation, migration, dendritic and axonal differentiation, cell death, collateral loss, synaptogenesis, and myelination (Faroon *et al.,* 2001). There are many studies suggesting that developmental neurotoxicants such as metals, pesticides, PCBs, or solvents can disrupt the function of adhesion molecules, cytoskeletal elements, ion channels, and other aspects of cellular regulation (Inglefield and Shafer, 2000). In addition, it is also possible that these toxicants may disrupt intracellular signal transduction pathways regulating transcription, translation, and post-translational modification of proteins (Margolis *et al.,* 1994). Toxicants may also disrupt developmental events by interfering with hormones, neurotransmitters, and their receptors (Margolis *et al.,*

1994). Some developmental neurotoxicants can increase oxidative stress, which may result in the aberrant stimulation of neuronal differentiation or programmed cell death (Mendola *et al.,* 2002). Little is known about the extent to which variations by toxicants affect normal brain development. A variety of toxicants may disrupt the same pathway. In other cases, a given toxicant may disrupt several different pathways. The effect of a toxicant on brain development may be the result of combinations of many different actions. Environmental factors such as nutrition, traumatic injury, infection, or exposure to multiple toxicants can alter the distribution of toxicants and the interactions of toxicants with specific targets in the developing brain (Squire, 2003).

One major mechanism guiding neural development is cell-cell and cell-matrix signaling processes mediated by cell surface proteins called adhesion molecules. Three types of these molecules have been identified such as cadherin, integrin, and immunoglobulin families. Among them, interruptions of cadherin by chemical insults have been investigated extensively and suggested as one of the important mechanisms for developmental neurotoxicities (Schettler, 2001). Cadherins are cell surface transmembrane glycoproteins mediating calcium-dependent intercellular adhesion and signaling interactions. A fundamental action of cadherins is to mediate neuronal adhesion and proper expression (Slikker and Chang, 1998). It is critical for early neural development, neurite growth, and synaptogenesis. Some studies have shown that neuroxicants disrupt cadherins during the developmental period (Castoldi *et al.,* 2001). Such effects could be either direct or indirect. One possible mechanism of direct inhibition could be through displacement of calcium ions by heavy metals such

as lead or cadmium (Carr *et al.,* 2002). Indirect inhibition includes alterations of calcium channels, interruptions of signal transduction pathway, and inhibition of cellular enzymes such as kinases, phosphatases, metalloproteases (Corey *et al.,* 1996).

C. Disruption of Intercellular Communication

The control of neurite growth depends on the interactions among the plasma membrane, adhesion molecules, their receptors, the cytoskeleton, intracellular calcium concentrations, and numerous signaling molecules (Furuyashiki *et al.,* 2002). Biochemical studies have shown that many neurotoxicants disturb one or more of these molecules (Costa *et al.,* 2001). Toxic effects on neurite development have been studied both *in vivo* and *in vitro*. During *in vivo* studies, embryos are usually exposed to toxicants *in utero* via the pregnant mother or postnatally via mother's milk and later in food or water (Mendola *et al.,* 2002) Anatomic, biochemical, and physiologic studies are then performed on the brains of the offspring. *In vitro* studies assess a variety of anatomic, biochemical, or physiologic parameters in neurons exposed to toxicants in cell culture. There are three known mechanisms whereby toxicants may alter neurite development: alterations of calcium-regulated processes, cytoskeletal structure and function, and protein phosphorylation (Stein *et al.,* 2002). Intracellular calcium plays significant roles in neurodevelopment. Some metals such as lead, mercury, and manganese disrupt intracellular calcium homeostasis and induce neurotoxicities (Mattson *et al.,* 1995). Microtubule-disrupting agents (e.g., nocodanazole, vinblastin, taxol) are representative toxicants inducing the death of neuronal cells by destruction of the cytoskeleton (Annau and Cuomo, 1988). In the central nervous system, many phosphoproteins are involved in various aspects of

brain development such as gene expression, protein synthesis, and cellular differentiation. There are three essential components to phosphorylation systems: a specific protein kinase catalyzing the phosphorylation reaction, a substrate protein and a protein phosphatase catalyzing the removal of the phosphate group. All of these components can be adversely affected by developmental neurotoxicants (O'Callahan, 1994).

D. Disruptions of Myelin and Neurotransmitters

Myelin greatly increases the efficiency of the nervous system, facilitating electrical conduction while conserving metabolic energy and space (Schettler, 2001). During development, myelin-forming cells produce a specialized extension of their plasma membrane. These cells invade the developing nerves, where they migrate along bundles of axons, proliferate, and segregate those axons individually (Bower and Bolouri, 2001). As they cease migrating, they synthesize a basal lamina, composed of laminin, merosin, collagen, fibronectin, and heparin sulfate proteoglycan. The segregated axons will be wrapped up by these cells. This process is called myelination. Myelination represents a complex series of metabolic and cellular events involving intercellular recognition and interaction, adhesion, synthesis, sorting and assembly (Bower and Bolouri, 2001). In general, myelination starts at the peripheral nerves, then the spinal cord, and finally the brainstem, cerebellum, and cerebral hemisphere. Myelination is especially susceptible to toxic insults, nutritional deficiencies, genetic disorders of metabolism, viral infections, and other environmental factors (Squire, 2003). Chemical insults during the proliferation of myelinating cells may be disruptive, leading to an irreversible deficit of myelin-

forming cells and permanent hypomyelination. Perturbation of myelination at a later stage may result in reversible deficits. Depending on the timing of the insult, myelin deficiency may result in the failure of myelin-forming cells to proliferate, the reduction of axonal development, and the decrease of myelin formation at the time of maximal synthesis.

Neurotransmitters play highly specialized roles in vertebrate and invertebrate nervous system (Gilbert, 2000). Neurotransmitters serve as developmental signals regulating basic cellular functions such as cell proliferation, differentiation, migration, and morphogenesis by receptor mediated mechanisms (Brown, 2000). These functions could make the developing nervous system especially vulnerable to environmental toxicants targetting neurotransmitter receptors (Bower and Bolouri, 2001). For example, PCBs are antagonists of GABA receptors (Edwards *et al.*, 2001). GABA acts as a trophic signal for many types of developing CNS. Neurotransmitters, growth factors, and cytokines play important roles in various aspects of nervous system development and they transmit their signals to the nucleus through second messengers. Second messengers may induce gene expressions for proliferation, differentiation, and maturation. Several studies have shown that some signaling pathways may be overexpressed or overactive during certain stages of neurodevelopment, enhancing the action of extracellular signals (Raeymaekers, 2002). Furthermore, these pathways are highly interactive and can modulate each other in a complex pattern. Interactions of various chemicals with specific signaling pathways have been demonstrated and potential impacts are being investigated (Bower and Bolouri, 2001).

E. Disruptions by Apoptosis and Biochemical Components

Apoptosis plays important roles in normal development, including the removal of embryonic structures that are unnecessary in the adult and the controlled removal of excess, nonfunctional or misplaced cells (Bower and Bolouri, 2001). Generally, the number of both neurons and oligodendrocytes formed at early stages exceeds the optimal number. Fifty percent or more of sensory neurons and motor neurons formed during development undergo apoptosis (Andersen *et al.,* 1987). Environmental stresses, including hyperthermia, radiation, genotoxic agents, chemotherapeutic drugs, reactive oxygen species, steroid hormones, and growth factor withdrawal have been shown to induce apoptosis. Exposure to apoptotic agents during development may induce congenital malformations (Andersen, 1989).

The early stages of development have been considered to be an "all or none" periods of susceptibility for congenital defects (Andersen, 1991). This is primarily due to the totipotent nature (i.e., the ability to give rise to all cell types) of the embryonic cells at these stages of development. Toxicants may produce a generalized disruption, leading to a developmental delay (Bois *et al.,* 1996). It is also possible that toxic agents act in a more specific manner on target areas during development. The final result will be subsequent failure of normal embryonic homeostatic properties. Neurotoxic insults during different developmental phases may produce diverse phenomena based on both the time and the duration of exposure. For example, exposure of metamphetamine to adult rats can induce the loss of dopamine and serotonin in the brain. However exposure of metamphetamine to developing rats does not produce the same effects in adult rats. Rather,

metamphetamine induces learning and memory deficits in developing rats. Different effects between adult rats and developing rats may be due to the maturational status of the CNS at the time exposure takes place (Byczkowski *et al.,* 1994).

Cellular proliferation and differentiation of cholinergic neurons occur with temporal and regional selectivity during pre- and post-natal development. Organometals such as triethyl-Pb and MeHg can induce the alteration of cholinergic neurons (Abbas and Fisher, 1997). Some organophosphate insecticides are neurobehavioral toxicants and are more toxic to the developing fetus than to the adults. Pyrethroid insecticides exert cholinergic effects in the developing brain, some of which are indicated by changes in acetyl choline esterase and muscarinic receptors (Parham *et al.,* 1997). PCBs affect the developing CNS, and their effects may persist into adulthood. One possible mechanism for the effects of PCBs is the deregulation of choline acetyl transferase (ChAT) activity by PCB-induced hypothyroidism (Kaufman and Thomas, 1987). During early brain development, the activation of NMDA receptors may play an important role in neuronal migration, elaboration of dendrites, synaptogenesis, synaptic plasticity, and cell survival. Some toxicants have shown the inhibitory effects on NMDA receptors (Kaufman and Thomas, 1987). During the postnatal period, rapid growth of the brain occurs. This stage is also associated with numerous biochemical changes that mature the fetal neonatal brain (Albert and Othmer, 2003). Interference by reactive oxygen species during this stage can cause several pathologic changes in the CNS. High levels of antioxidant enzymes during CNS development have been postulated to protect against oxidative damage.

Several neurotoxicants can induce oxidative stress and/or inhibit the activities of antioxidant enzymes (Raeymaekers, 2002).

III. Perinatal Transfer of Chemicals

Most of the information described below is based on the research from human studies. However, other mammals, like rodents and monkeys, have similar characteristics and time course as humans.

A. Organ Growth and Body Weight Changes

Fetal weight increases dramatically during the gestational period and is affected by maternal age, weight, race, ethnicity, fetal sex, and the number of fetuses (Andersen and Dennison, 2001). Growth retardation may occur due to a number of factors, including the nutrition of the mother and subsequent nutrient supply available to the developing fetus. Placenta grows during same period but shows a pattern distinct from fetal growth. (Ureta-Vidal *et al.,* 2003).

The maternal component of total weight gain comprises the increases in uterine and breast tissue, extracellular fluid, and fat (Bunney *et al.,* 2003). The uterus shows a dramatic increase in cavity volume. Extracellular fluid is increased too. The increase of plasma volume accounts for approximately half of that; the remainder is interstitial fluid. Sometimes, this can induce edema in pregnant women. The major component of maternal weight gain is accumulation of fat. Maternal fat stores are laid down primarily during the first half of pregnancy and provide an energy store for the third trimester when fetal growth predominates.

B. Physiological and Biochemical Changes during Pregnancy in the Mother

There are many physiological and biochemical changes to maintain a pregnancy. Here, we will briefly overview physiological changes during pregnancy focusing on the human. First, changes in the endocrine system occur (Williams, 1999). In the placenta, human chorionic gonadotrophin (hCG) and human prolactin (hPL) are produced. They are important in maintaining pregnancy and growth. Production and secretion of progesterone, estrogen, cortisol, aldosteron, adenocorticotrophic hormone (ACTH), and melanocyte sitmulating hormone (MSH) increases throughout pregnancy. In addition, the production of thyroxine,tri-iodothyronine, and parathyroid hormone increases. The increase of parathyroid hormone will induce calcium absorption by the gut, resulting in the maintenance of calcium supply to the fetus.

Second, there are changes in maternal metabolism (Kennedy, 2002). The fasting plasma concentration of glucose falls during the first trimester, then rises and falls again towards term. Furthermore, there seems to be a progressive reduction of glucose tolerance following an oral glucose load. The plasma concentration of free fatty acids, glycerol, cholesterol, and phospholipid increases during pregnancy. The significances of these changes are not clear but they should lead to increases in the transplacental transfer of these substrates to the fetus. Pregnancy causes a reduction in amino acid catabolism because amino acids are used preferentially for gluconeogenesis. Plasma albumin amount decreases during pregnancy, contributing to the edema seen in late pregnancy.

Third, cardiovascular changes occur (Honore, 2001). Plasma volume increases by approximately 43%, resulting in the physiological anemia of pregnancy. Cardiac

output increases by approximately 40% during the first trimester because of an increase in both heart rate and stroke volume. Much of the increased cardiac output is directed to the uteroplacental circulation, which increases blood flow by 10 times.

Fourth, changes in the respiratory system and renal function occur (Honore, 2001). Due to fetal oxygen consumption, oxygen demand increases. This can be met by increased red cell mass and ventilation. Excretion of glucose, water-soluble vitamins, and amino acids increases. These changes are partially due to the increase of glomerular fitration rate (GFR) and diminished ability for reabsorption.

C. Physiological Changes in Fetus and Neonate

Before birth, the lungs in the fetus are not functional and blood flow is diverted away from them. Oxygen and nutrients are all transferred from the the placenta. When the baby takes its first breath, pulmonary blood flow starts (Sabounchi-Schutt *et al.,* 2000). Cardiac output is a meaningless term in fetal life because of the shunting of blood. In the neonate, cardiac output is higher than that in the adult. Heart rate may rise at birth but falls after that, reaching adult rates at puberty (Ureta-Vidal *et al.,* 2003). The number of red cells increases dramatically throughout gestation and in general the sizes of cells are bigger than those of adults. After birth there is a rapid breakdown of a number of red cells (Aebersold and Goodlett, 2001).

The fetus obtains all nutritional requirements from its mother via the placenta. Glucose, amino acids, and fatty acids cross the placenta easily, but the passage of some lipids is restricted. Plasma glucose level is half that of adults but its control is poor. Fetal plasma concentration of amino acids is higher than in adults, which is considered due to active transport mechanisms from the placenta. Towards the end of

gestation, relatively large amounts of glycogen are stored in fetal muscle, liver, and fat. This energy source will be used for the survival of the neonate before feeding is initiated (Lin *et al.*, 2003).

D. Mechanisms of Chemical Transfer through Placenta

The placenta, which is vital for normal fetal growth and development, has three main functions. First, the placenta is an important endocrine organ of pregnancy. Second, the placenta protects the fetal allograft from attack by the maternal immune system. Third, the placenta is the interface between maternal and fetal plasma across which maternofetal transfer of nutrients and waste products occurs (Aebersold and Goodlett, 2001).

The placenta shows the greatest morphological and physiological diversity across species. For example, pig and sheep placentas have four continuous cell layers separating the maternal and fetal plasma whereas the human placenta has two layers (Aebersold and Goodlett, 2001). Therefore, the permeability of sheep and pig placentas to solutes is lower than that of the human placenta. The two cell layers separating maternal and fetal blood in the human placenta are the syncytiotrophoblast and the fetal capillary epithelium. Solutes may cross these cell layers by passive diffusion, carrier mediuated transport, and endocytosis/exocytosis (Romijn *et al.*, 2003).

Passive diffusion. Fick's law of diffusion can be related to placental transfer of hydrophobic molecules. Since the fetal side is slightly negative from the maternal side in terms of ionic composition, positively charged materials will be transferred to the fetus in addition to hydrophobic solutes.

Carrier-mediated transport. Transcellular transport across the syncytiotrophoblast requires two transport proteins, one in the maternal-facing plasma membrane and the other in the fetal-facing plasma membrane. Ionized calcium can be transferred by this mechanism.

Endocytosis/ exocytosis. Large molecules (e.g., proteins) may gain access into the placenta by fluid phase endocytosis in which the plasma membrane invaginates to engulf the solutes in the extracellular space. In receptor-mediated endocytosis, the solute binds to specific receptors on the plasma membrane, which eventually invaginates. The syncytiotrophoblast of the placenta is well endowed with coated pits and vesicles, which are indispensable for endocytosis. Thus, this may be the primary mechanism of maternofetal transfer used for large molecules such as immunoglobulins.

Transport of chemicals to the embryo/fetus occurs across both the yolk sac and chorioallantoic placentas. Chemicals with low molecular weight (< 500 Da) or high lipophilicity can be easily transferred to the embryo/fetus. Ionizable agents can cross the placenta. However the degree of ionization is important for the placental transfer and subsequent accumulation in the fetus. Thus, the relative pH of maternal and fetal tissues affects the degree of placental transfer. Protein binding in maternal plasma is also an important determinant of embryo/fetal availability. Agents that are bound to maternal plasma proteins are not available for placental transfer.

E. Mechanisms of Chemical Transfer through Breast Milk

In late pregnancy, progesterone and estrogen play a role in stimulating the growth of mammary glands in order for the milk to be secreted (Honore, 2001). At

parturition, dramatic changes occur in the mammary glands, leading to milk secretion. Milk is produced and stored in alveolar units in mammary glands (Linzell and Peaker, 1975). Removal of milk from alveoli is accomplished by contraction of myoepithelial cells surrounding the alveoli. The production, secretion, and composition of milk are determined by endogenous hormones such as progesterone, estrogen, growth hormone, adrenal glucocorticoids, and insulin (Corley *et al.*, 2003).

Five distinct pathways are involved in the synthesis and secretion of milk in mammary glands (Oskarsson *et al.*, 1998). Even though the composition of milk is different from one species to another, the biochemical mechanisms by which the various milk components are secreted seems to be similar.

Exocrine pathway. Most of the components in the aqueous phase of milk are secreted by this pathway. The major milk proteins are synthesized on ribosomes and transferred to the Golgi system. Secretory vesicles produced in the Golgi system move to the plasma membrane where they fuse and release their contents into the milk space. This process appears to be largely constitutive.

Lipid transport pathway. Milk lipids are secreted to the mammary glands via the unique pathway. Triglycerides are synthesized from precursor fatty acids in the smooth endoplasmic reticulum of the alveolar cell and coalesce into large droplets that are drawn to the apex of the cell. The droplets become enveloped in apical membrane and are then extruded from the cell to form the milk fat.

Transport across the apical membrane. Small molecules including sodium, potassium, chloride, and glucose can pass across the apical membrane. The detailed mechanism of this pathway still remains to be explored.

Transcytosis. Intact proteins can cross the mammary epithelium from the interstitial fluid by transcytosis. Immunoglobulins are the best studied molecules that pass through transcytosis. The proteins bind to receptors at the basolateral membrane. At the apical membrane the extracellular portion of the receptor is cleaved and secreted with bound protein. Many peptide hormones and proteins are considered to be transferred via this pathway.

The paracellular pathway. During lactation the passage of molecules between alveolar cells is impeded by a gasket-like structure called the tight junctions. Sometimes the junctions become leaky, allowing the milk components of interstitial space to pass into the milk.

The transport of chemicals into milk is thought to follow the same pathways as do milk components. However, the real mechanisms of chemical transfer into the milk have not been thoroughly investigated. In general, passive diffusion across the membrane is belived to be the major transport mechanism of xenobiotics (Corley *et al.,* 2003). Unionized and lipophilic chemicals can be transported by this way. Thus, the pKa of the chemical and the local pH can be important factors determining the lactational transfer of chemicals. Chemicals can also bind to proteins, which are more important in plasma than in milk, or associate with lipid droplets suspended in milk, depending on their molecular weight and octanol:water partition coefficients. In addition, some type of carrier proteins may be involved in the transfer of toxic metals into the milk (Oskarsson *et al.,* 1998).

IV. Toxicology of Chemical Mixtures

A. Overview

In real environment, people are exposed to chemical mixtures everyday. Hundreds of synthetic chemicals contaminate our environments. Multichemical exposures are ubiquitous, including air and soil pollution from municipal incinerators, leakage from hazardous waste facilities, uncontrolled waste sites, or the contaminants in drinking water (ATSDR, 1999). Complex mixtures from the same source can vary considerably. For example, automobile exhaust varies dramatically among sources and over time. The composition of air pollution varies with time, geographic location, and source. In addition, many complex mixtures such as automobile exhaust, and cigarette smoke, have hundreds or thousands of chemical components. However, almost all toxicologic tests have focused on one chemical at a time. This raises important questions concerning interactions among chemicals. Since the numbers of chemical combinations are so large, it is beyond the capacity of modern science to test all mixtures, or even all common mixtures. Thus, one of the most important issues in toxicology is to develop efficient means to predict toxic interactions in chemical mixtures. Chemicals will interact in one of three ways: additive effect, synergistic effect, or antagonistic effect. Additive effect represents that the effect of chemical mixture is the sum of the effects of each chemical. The National Academy of Sciences concluded that effects of exposures to agents with low response rates usually appear to be additive (NAS, 1983). Synergistic effect means that the net effects of chemical mixture are higher than the sum of the effects of each chemical. If the individual components of a chemical mixture have different biological mechanisms of toxicity, the net effect may be synergistic (Jonker *et al.,* 1990; Groten *et al.,* 1994). Antagonistic effect shows that the net effects of chemical mixture are

lower than the sum of the effects of each chemical. If the chemicals have same target but different mechanisms of action, antagonism is the usual outcome (Jonker *et al.*, 1993).

Most of the information available on interactions among chemicals comes from human occupational studies and from rodent toxicological studies. Those studies generally evaluate doses that are much higher than environmental exposures commonly encountered. For the most part, exposure to chemical mixtures in the environment occurs at "low" doses. Specially, dose is important in the characterization of mixture effects as interactive effects depend heavily on dose. Therefore, more mechanistic studies are needed to derive useful information regarding the toxic potential of chemical mixtures. In addition to biological approches, other areas such as the application of statistical methods, physiologically based pharmacokinetic/ pharmacodynamic (PBPK/PD) modeling, and the use of science-based risk assessment approaches have been used to investigate the interactive effects of chemical mixtures. More advanced discussions on these areas were summarized recently by Yang et al. (2004).

The area of chemical interactions can be divided into two major categories: pharmacokinetic interactions and pharmacodynamic interactions. Pharmacokinetic interactions represent the changes of chemical concentrations in the tissues by co-exposure with other chemicals. Pharmacodynamic interactions represent the changes of chemical effects on the tissues or cells by co-exposure with other chemicals. In the following sections, brief discussion of each category will be described.

B. Pharmacokinetic Interactions of Chemical Mixtures

Pharmacokinetic interactions involve alterations in absorption, metabolism, excretion, or disposition of a chemical (Lee *et al.*, 2002). These interactions can be mediated by the induction or inhibition of enzymes and binding proteins. The following picture summarizes the general types of pharmacokinetic interactions.

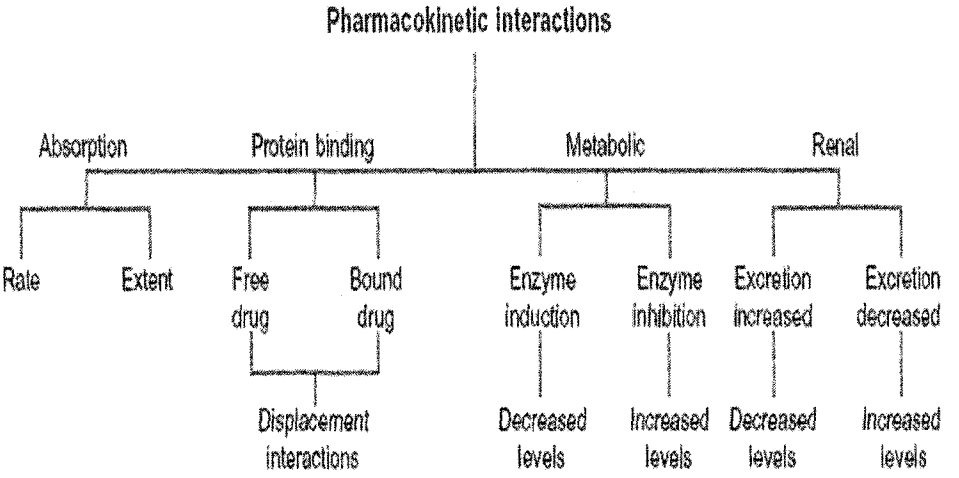

Fig. 1.2. Types of pharmacokinetic interactions.

Even though many pharmacokinetic interactions between drugs or chemicals have been shown, the underlying mechanisms for interactive effects are not comprehensive. The research on pharmacokinetic interactions between chemical mixtures is in its infancy partly because the net effects between chemicals depend on the combination of each chemical, which may be quite different from site to site. Therefore, it is recommended that the approach can incorporate mechanistic considerations on all possible interactions and estimate the effects according to diverse exposure scenarios. Computational modeling is attractive to researchers because it has the power to incorporate diverse biological information and provide simulation results according to exposure scenarios. Among many computational modeling techniques, PBPK modeling has been adopted and used in the studies

investigating pharmacokinetic interactions. Some studies have shown the application of PBPK modeling to investigate pharmacokinetic interactions in chemical mixtures (Yang *et al.*, 2004). These studies included the mechanistic considerations regarding metabolism and disposition and extended their contribution from simple binary mixtures to complex mixtures such as gasoline.

C. Pharmacodynamic Interactions of Chemical Mixtures

Pharmacodynamic interactions include the change of chemical effects on a tissue's response or susceptibility by co-exposure with other chemicals (EPA, 1999). These interactions can be caused by depletion or induction of protective factors, alterations of tissue response, changes in hemodynamics and immunomodulation. Predicting the toxicity of a complex mixture can be assisted by testing it in bioassay systems and comparing the results with those of similar mixtures of known toxicity or risk. Useful bioassays for testing mixtures could range from mutation tests in microorganisms to evaluation of effects on organs in culture or short-term tests in rodents (EPA, 1999). Recently, genomics/proteomics have been used in the research on chemical mixtures (Yang *et al.*, 2004). At the cellular level, chemicals can exert their toxicities by direct or indirect alterations in gene expression. Disruption of important signal transduction pathways mediates the toxicity of many chemicals (Thomas et al., 2001). Some studies have demonstrated that chemicals with similar toxicological effects induce gene expression alterations in a common battery of genes (Hamadeh et al., 2002; Thomas et al., 2001). DNA microarray studies for chemical toxicants showed 5 distinct expression profiles depening on the mechanisms of toxicities (i.e., peroxisome proliferators, aryl hydrocarbon receptor agonists,

noncoplanar PCBs, inflammatory agonists, and hypoxia-inducing agents). Toxicological studies showed the potential utility of gene expression profiling by identifying a common suite of genes altered during malignant transformation of human keratinocytes by diverse chemical carcinogens (Bae et al., 2002, 2003).

In addition to biological assays, computational modeling can contribute to the estimation of the interactive effects. The linkage of computational modeling with experimental toxicology of chemical mixtures will have great potential in application to risk assessment for chemical mixtures. Recently, the linkage of PBPK modeling and Reaction Network Modeling highlighted the potential of generic simulation platform for complex biological systems involving chemical mixtures and/or multiple stressors (Yang *et al.,* 2004). They suggested that reaction networks with overlapping metabolic reactions of many xenobiotics or reaction networks of signal transduction pathways in cellular systems under the influence of environmental pollutants may be linked to the liver compartment of a PBPK model. These findings suggest that pharmacodynamic interactions can be incorporated into a PBPK model, generating more advanced PBPK/PD model.

V. Developmental Neurotoxicology of MeHg and PCBs

A. Introduction and Human Exposure

MeHg and PCBs represent two of the frequently encountered environmental contaminants during the developmental period. Developing organisms are often exposed to these chemicals concurrently. Mercury exists in a number of different chemical forms but is usually released into the environment as a metal or inorganic compound (Fitzgerald and Clarkson, 1991). Major sources of mercury are coal-fired

power plants and waste incinerators. Currently, human exposure to MeHg is mainly through fish consumption. Mercury compounds are generally released into the environment as the inorganic form from both natural sources and anthropogenic activities, such as mining (Fitzerald and Clarkson, 1991). Inorganic Hg compounds can then be converted into the organic form of mercury, such as MeHg, via microbial action and can be further accumulated in fish. Mercury in sediments and river or ocean water is also converted into methyl mercury by bacteria, which bioaccumulates as it passes up the food chain (Renzoni *et al.*, 1998). As a result, pregnant and lactating women consuming fish may be exposed to methyl mercury at levels that pose a threat to the developing brain of the fetus and newborn babies. Forty states in United States have issued fish-advisory warnings for the women of reproductive age to limit or avoid fish consumption as a result of concensus over mercury contamination (ATSDR, 1996).

Historical endemic disasters in Japan and Iraq have taught us the devastating effects of MeHg exposure on the developing CNS (Bakir *et al.*, 1973; Harada, 1977). From these studies, it has been shown that the developing CNS is particularly vulnerable to the effects of MeHg since the neurodevelopmental effects were observed when the mothers of the affected children did not show toxicity. The developmental effects of MeHg exposure encompass a wide range of behavioral changes. Several effects observed in humans such as visual and auditory deficits, impairment of motor function, and visual recognition memory, are reproduced in primates following *in utero* MeHg exposure (Burbacher *et al.*, 1990). Pathological examination of brain tissues following moderate MeHg exposure *in utero* include

disorganization of cell layers, disoriented cells and cell loss in all the species examined (Burbacher *et al.*, 1990). Biochemical mechanisms have been proposed to explain the observed pathological features associated with MeHg exposure, including alteration of calcium homeostasis, mitochondria function, inhibition of protein and DNA synthesis, inhibition of neuronal transmission and cell cycling, mitotic arrest, and oxidative stress (Atchison and Hare, 1994; Ou *et al.*, 1997; Ponce *et al.*, 1994).

PCBs are industrial chemicals that were used for a variety of purposes, such as lubricants, coatings, and insulating material for transformers. Although PCB production has been banned because of their environmental persistence, they still exist in the environment (ATSDR, 1999). PCBs are a family of chlorinated hydrocarbons containing 209 different congeners. PCB congeners that can assume a co-planar configuration are capable of binding to the aryl hydrocarbon (Ah) receptors and this can initiate biological actions and toxicity similar to Ah-receptor agonists such as 2,3,7,8 -terachlorodibenzo-dioxin (TCDD) (Safe, 1984). In contrast, congeners possessing chlorine substitution at the *ortho* positions of the biphenyl rings are non-planar and do not bind to Ah receptor (Kafafi *et al.*, 1993). The toxicological significance of PCB exposure became known after accidental exposure in Japan and Taiwan (Hsu *et al.*, 1985; Kuratsune *et al.*, 1971). Subsequently, several epidemiological studies documented that low-level, background exposure to PCBs is associated with adverse effects on various neurodevelopment parameters in humans (Huisman *et al.*, 1995; Jacobson *et al.*, 1990). Developmental exposure of several laboratory species to PCBs resulted in effects such as alteration in sensory and cognitive functions, persistent increase in motor activity, and behavioral changes

(Tilson *et al.*, 1990). At the cellular and molecular levels, developmental exposure to PCBs has shown to disrupt thyroid hormone homeostasis and alter dopamine levels in the brain (Goldey *et al.*, 1995; Seegal *et al.*, 1997).

Exposure to PCBs is largely through dietary sources, including oral ingestion of contaminated dairy products, meat, or breast milk, all of which contain a mixture of non-coplanar and co-planar congeners (Safe, 1984). PCBs tend to bioaccumulate in fat tissues (Carpenter, 1998). Therefore, concentrations of PCBs are highest at the top of the food chain, including beef, pork, dairy products, and fish. PCBs are easily passed during pregnancy from mother to fetus, and continue to be transmitted during the breast feeding period (DeKoning and Karmaus, 2000). Maternal fish consumption represents an important pathway for co-exposure to both MeHg and PCBs. Present levels in some areas including the Great Lakes in the U.S. exceed the reference doses for both MeHg and PCBs (Rice, 1995). Interactive effects among PCB congeners and related compounds on various biological endpoints have been observed. For example, using hepatic cytochrome P450 induction, hepatic tumor promotion, and immunotoxicity as endpoints, non-planar PCBs are shown to enhance or suppress the biochemical and toxic response elicited by TCDD in rodents (Smialowitz *et al.*, 1997; van Birgelen *et al.*, 1996; Wolfle, 1997). Non-additive interactions between co-planar PCB 126 and PCB 153 as promoters of hepatic preneoplastic lesions in rats have been observed (Haag-Gronlund *et al.*, 1998). The interactions of PCBs on developmental or CNS toxicity have also been shown (Borlakoglu *et al.*, 1992). In addition, co-administration of PCB 153 with TCDD

results in the increase or decrease in the incidence of cleft palate induced by TCDD treatment alone (Morrissey *et al.*, 1992; Birnbaum *et al.*, 1985).

B. Mechanisms of Developmetal Toxcities by MeHg and PCBs

MeHg and PCBs are known to induce functional deficits in the brains of babies and children exposed to these chemicals during the gestational and/or lactational period (Trask and Kosofsky, 2000). However, the modes of actions are still uncertain even though many mechanistic studies for both toxicants have been conducted. We summarize the suggested mechanisms of developmental neurotoxicities by MeHg and PCBs in Table 1.1 presented at the end of this chapter.

C. Possible Mechanisms for Interactive Effects between MeHg and PCBs

Numerous studies have been reported that both MeHg and PCBs interfere with biological processes critical for CNS development. For example, maternal exposure to PCBs resulted in an alteration of NMDA-dependent long-term potentiatiation (LTP), a cellular model of synaptic plasticity, which may be representative of learning and memory processes in the mammalian brain (Altmann *et al.*, 1995). Pre- and postnatal exposure to PCBs altered levels of several neurotransmitters (Eriksson *et al.* 1993; Tilson and Kodavanti, 1997), while MeHg was shown to interfere with synaptic transmission and membrane excitability (Yuan and Atchison, 1993; Yuan and Atchison, 1995). PCB congeners have also been shown to affect protein kinase C (PKC) activation/ translocation *in vitro* and *in vivo*. Both PCB congeners and MeHg have been shown to disrupt Ca^{2+} homeostasis, a critical component for proper cellular and neuronal functions (Bemis and Seegal, 2000). Both coplanar and non-coplanar PCBs increased intracellular Ca^{2+}, but only the non-planar PCB congeners interfere

with sequestration and extrusion mechanisms that reduce intracellular Ca^{2+} levels (Kodavanti and Tilson, 1997). MeHg has been shown to increase intracellular Ca^{2+} levels with concommitant increases in Zn^{2+} (Denny and Atchison, 1996; Hare *et al.*, 1993; Marty and Atchison, 1997). Both MeHg and PCBs affect cognitive development in animals exposed *in utero* (Burbacher *et al.*, 1990; Grandjean *et al.*, 1997; Schantz *et al.*, 1995; Tilson *et al.*, 1990). Interactive effects between MeHg and PCBs have been shown in some studies. The presence of neurobehavioral deficits in children exposed to the highest amount of mercury and PCBs through a perinatal route indicated a possible interaction between MeHg and PCBs (Grandjean *et al.*, 2001). Co-exposure to MeHg and PCBs affected cytosolic calcium homeostasis in a non-additive manner (Bemis and Seegal, 2000) and reduced dopamine content in brain synergistically (Bemis and Seegal, 1999). These results suggest that co-exposure to MeHg and PCBs through perinatal exposure may affect normal brain development in a non-additive manner, resulting in functional deficits in babies and children. Given the similarities between two agents regarding distal learning and memory defects and proximal effects on calcium signaling, it is likely that MeHg and PCBs interactively alter cognitive development.

VI. Biologically-based Computational Modeling: PBPK Modeling and Boolean-Network Modeling

A. Overview of Biologically-based Computational Modeling

In general, biological researches have focused mainly on individual molecules, on their properties as isolated entities or as complexes in very simple model systems. However, biological components in the body participate in very complex networks,

including regulatory networks for gene expression, intracellular metabolic networks and both intra- and intercellular communication networks. Groups of genes form regulatory networks with complex behaviors. These networks control other genes catalyzing specific biochemical reactions, as well as the small molecules which are substrates or products of these reactions (Bower and Bolouri, 2001). Genetic regulation can indirectly control biochemical reactions in cellular metabolism. Also, cellular metabolism itself can regulate gene expression. Such networks are also involved in homeostasis and differentiation of cellular systems. Thus, it is crucial to construct a platform integrating complex biological components coherently into the overall living system. That is, the interdependent biochemical processes should be interpreted and analyzed in terms of complex dynamical networks (Bower and Bolouri, 2001). In this perspective, computational modeling will be necessary. Through the modeling of biological networks, all possible links can be connected to each other to form the system. One of the obstacles in constructing computational model is the lack of extensive biological information. However, the development of genomics and proteomics makes it more feasible to explore the comprehensive biological information. In addition, molecular biology begins to turn towards informatics and systematically collect the results relating to biological information. These data have been, and will be, stored systematically in specific databases, which are accessible via the internet. There are databases available via the internet for all known sequenced genes (e.g. EMBL), proteins (e.g. SWISS-PROT, PIR, BRENDA), transcription factors (TRANSFAC), biochemical reactions (KEGG) and signal transduction pathways (TRANSPATH, GeneNet) (Brown, 2000). Therefore, the

environment for biologically-based computational modeling is better than ever. At tissue or organ levels, the concepts and applications of PBPK modeling provides a good illustration of computational simulation in biological sciences. At cellular or subcellular (i.e., gene and protein) levels, many approaches are being explored these days, including stochastic models, petri, boolean networks, the logical approach, threshold models, and approaches based on differential equations (Bower and Bolouri, 2001). In the following subsections, two representative computational simulation, PBPK modeling and Boolean-network modeling, will be discussed.

B. PBPK Modeling

PBPK modeling is a powerful tool for the estimation of a target tissue dose involved in the disposition of the chemical of interest and the correlation of the chemical absorption, distribution, metabolism, and excretion among the organs and tissues after chemical exposure (Andersen *et al.,* 1987). A PBPK model is composed of known physiological parameters (blood flow rates, tissues volumes, breathing rates, etc.), chemical-specific parameters (partition coefficients, chemical density, metabolic constants, molecular weight, etc.), and species-dependent parameters (Yang *et al.,* 2004). A graphical scheme is usually formed to represent the physiological tissues and organs. Mass balance equations describe the body mathematically with appropriate parameters carrying biological significance. Each equation describes the "in-out" relationship of chemical transfer in the tissue compartment. A series of equations representing all of the interlinked compartments is formulated to express the entire biological system mathematically. This model can then be used for computer simulation to predict the time course behavior of any given

parameter. Most physiological parameters are available in relevant literature such as the special report on the compilation of physiological parameters for PBPK models (Brown *et al.*, 1997). If some of the data are missing or information gaps exist, experiments measuring the parameters *in vitro* or *in vivo* can be done. The metabolic rates in a model can be adjusted to account for enhanced (or decreased) metabolism of a particular compound (Abbas and Fisher, 1997). In some cases, allometric extrapolation can be applied to the modeling (Lindstedt, 1987). A PBPK model can be developed and validated against the available experimental animal data, and then extrapolated to predict concentrations of toxic compounds in human tissues (Andersen, 1989). In addition, a PBPK model can be used to estimate target doses according to diverse exposure scenarios and each person's unique physiological and/or metabolic characteristics (Andersen, 1991; Bois *et al.*, 1996). Another advantage of PBPK modeling is that the physiological or pathological changes of the body can be incorporated into the model and used to predict the disposition changes of the chemicals depending on the body state. For example, a pregnancy model could accommodate changes in blood flow and tissue size and predict the concentration of the chemical in a developing fetus (Byczkowski *et al.*, 1994).

C. Boolean-Network Modeling

Rapid progress in the genomics of humans and other organisms led to the discovery of hundreds of genes whose function is to control and regulate the activity of other genes. These genetic networks comprise a complex system of feedback which ultimately controls the expression of genes. Genetic networks have been used to model biological systems for several decades. For computational reasons, the only

model system that has yielded insights into the overall behavior of large genetic networks (up to 100,000 genes) is the Boolean network model introduced by Kauffman (Kaufman and Thomas, 1987). Boolean networks constitute a model as follows. First, each gene may receive one or several inputs from other genes. Several inputs may be required to regulate one gene (*multigenic* regulation), and a particular gene may also be able to affect the expression of a wide variety of downstream genes (*pleotropic* regulation). Second, each gene can be modeled as a binary element (i.e., ON or OFF). Third, the output will be computed from the input according to logical or Boolean rules. Logical or Boolean rules compute its value based on the values of the elements connected with the target. The state of the system is defined by the pattern of states (on/off or 0/1) of all its elements. All elements are updated synchronously, moving the system into its next state, and each state can have only one resultant state. The state of the system is computed in discrete time steps. Therefore, at any moment (t) it is defined as follows: $S(t+1)=f(S(t),\{I1...In\},\{B1...Bn\})$. Since the number of all possible states of the system is limited, the system reaches a cycle or attractor. It can be a steady state (point attractor) or a limit cycle (dynamic attractor). Attractors can represent the target area of the organism as diverse phenotypes (e.g., cell types of development, repaired tissue following the response to injury, or the adaptation of metabolic gene expression). Until now, gene regulations, including transcription factors, have been modeled by Boolean network (Friedmann *et al.,* 1998). A model describing the interactions of proteins with regulatory DNA elements is established to explain the control mechanism of gene expression (Raeymaekers, 2002). In this model, protein elements are described as

activators (enhancers, facilitators) or inhibitors (suppressors, silencers). These elements are able to interact with specific segments of DNA or other regulatory proteins. In another model, the combinatorial nature of gene regulation has been modeled by Boolean network and confirmed in experiments characterizing multi-input control of gene expression in bacteria and fruit fly (Albert and Othmer, 2003).

VII. Proteomics

A. Overview

Genomics can be defined as the extensive exploration of genetic information, DNA. Genomic sequence data of many prokaryotic and eukaryotic cells has been summarized and categorized (Andersen and Dennison, 2001). Through the collaboration of life science with computer science, genomic sequence databases help researchers to analyze their DNA and protein sequence information biologically and statistically (Ureta-Vidal *et al.,* 2003). Other molecular biological techniques such as reverse transcriptase polymerase chain reaction (RT-PCR) can make mRNA levels monitored in real time. The meaningful progress in this area is the effort to connect the DNA sequence information and its mRNA expression level (Bunney *et al.,* 2003).

Proteomics is the complementary term to genomics. The term proteome, refers to proteins that are encoded and expressed by a genome, and was first suggested by Marc Wilkins (Williams, 1999). He defined proteomics as the extensive study of proteins. In other words, proteomics deals with how proteins are modified, when and where they're expressed, how they're involved in the metabolic pathways and how they interact with each other. Since the proteome shows dynamic changes in a cell with the response to extra- and intra-stimuli, the study of the proteome needs state-of-

art technologies such as mass spectrometry, laser science, nanotechnology, and high pressure liquid chromatography (Kennedy, 2002). Proteome analysis was originally derived from a protein characterization method, but the identification of proteins which are differentially expressed in response to a cellular activity is more powerful when it is combined with genomics. In general, proteomic research is composed of four areas: (1) expression proteomics is the identification of proteins involved in the special biological events and characterization of identified proteins; (2) quantitative proteomics is the quantitation of differentially expressed proteins in response to environmental challenges; (3) post-translational proteomics is the identification of post-translational modification such as phosphorylation, glycosylation; (4) protein-protein interactions include the investigation of structural and functional actions. Proteomics has been used in drug development, pharmacological and pathological studies (Honore, 2001). The applications of proteomics have become more widespread lately because of the rapid development of technologies and the comprehension of protein databases.

B. Two-dimensional Electrophoresis

One of the robust methods in proteomics is two-dimensional electrophoresis (Honore, 2001). Two-dimensional electrophoresis consists of isoelectric focusing (IEF) and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It has been a standard technique for the separation of complex mixtures of proteins. Typically, two-dimensional electrophoresis can separate up to thousand of proteins from whole cell or specific tissue homogenates.

IEF relies on the fact that each protein has a unique primary structure determined by the composition of polar and non-polar side chains of amino acids. Due to the different composition of amino acids, every protein has its own isoelectric point (pI). The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and the amino- and carboxyl-termini. The pI is the specific pH where the net charge of the protein is zero. If two different proteins are mixed in a pH gradient and subjected to the influence of an electric field, the proteins will migrate to the position in the gradient at which the net charge of each protein is zero, and will be separated from each other regardless of their molecular weight. For successful separation of protein mixtures by IEF, three parameters should be taken into account in the separation process: pH gradient, denaturation of proteins, and matrix. The degree of denaturation and solubilization of a protein is the most important factor to achieve successful separation of protein mixtures. Protein solubilization and denaturation need a mixture of urea and detergents so that each protein is only in its primary structure and minimizes aggregation or interaction among proteins. The pH gradient is formed either by carrier ampholyte in a tube gel matrix or with an Immobilised system. Due to inconsistencies in the resulting data, the use of carrier ampholytes is less widely used. Instead, Immobilised pH gradient (IPG) replaced carrier ampholytes beginning in 1982. An IPG is created by covalently incorporating a gradient of acidic and basic buffering groups (acrylamido buffer) into a polyacrylamide gel when it is cast (Sabounchi-Schutt *et al.*, 2000). IEF with IPG is more reproducible because the covalently attached gradient cannot drift.

The development of the IPG technique significantly improved the feasibility of IEF and protein separation.

SDS-PAGE separates proteins according to their molecular weights. SDS renders all proteins negatively charged, so that their charge cannot affect their migration in a ploy acrylamide gel within an electric field. SDS can also play a role in dissolving hydrophobic molecules. Thus all proteins retain only their primary structure and have a large negative charge which means they will all migrate towards the positve pole (Ureta-Vidal *et al.,* 2003). The denaturations of proteins are carried out by disrupting hydrogen bonds, blocking hydrophobic interactions, and unfolding the protein molecules. Denaturation of proteins is important because it minimizes differences in molecular form by eliminating the secondary and tertiary structure of proteins. To obtain complete protein unfolding, either dithiothreitol (DTT) or β-mercaptoethanol is added to reduce all cysteine residues, resulting in the breaking all disulfide bonds in the tertiary peptide structure. In this environment, proteins will all move towards the positive pole at the same rate, with no separation by size. Therefore it is needed to put the proteins into an environment allowing different sized proteins to move at different rates. The environment of choice is polyacrylamide. A polyacrylamide gel is not solid, but is made of tunnels through a meshwork of fibers. If all the proteins enter the gel at the same time and have the same force pulling them towards the other end, small molecules can manuver through the polyacrylamide forest faster than big molecules, which finally separate proteins by size.

C. Applications of Mass Spectrometry in Proteomics

The advancement of mass spectrometry technologies has changed proteomics. Mass spectrometry has essentially replaced the classical technique of Edman degradation as it is much more sensitive and offers much higher output (Aebersold and Goodlett, 2001). Two main approaches are employed using mass spectrometry for protein identification. In the 'peptide-mass mapping' approach, the mass spectrum of a peptide mixture is analyzed to show the peptide-mass fingerprint of the protein. This is usually achieved by matrix-assisted laser desorption ionization- time of flight mass spectrometry (MALDI-TOF) (Lin *et al.,* 2003). MALDI utilizes nitrogen lasers for the ionization of biomolecules. Incorporation of an analyte into the crystalline structure of small UV-absorbing molecules provides a safe vehicle for ions to be created from polar or charged biomolecules. MALDI Time-of-Flight (MALDI-TOF) has become the most feasible and accurate tool to obtain the mass fingerprint of a peptide mixture. Mass fingerprint information can be searched against protein databases for identification and post-translational modification. The second method for protein identification relies on the fragmentation of individual peptides in the mixture to gain sequence information. In this method, the peptides are ionized by electrospray ionization (ESI) directly from the liquid phase (Aebersold and Goodlett, 2001). ESI is a continuous ionization method that creates molecular ions by a potential difference between a capillary and the inlet to the mass spectrometer. Charged droplets are generated by the electric field in the form of mist. The important advantage of ESI is the ability to form highly charged ions without further fragmentation. It lowers the mass-to-charge (*m/z*) values to a range easily measured by various types of mass analyzers. From this simple division of a measured mass

with its true charge status, the calculation of the true molecular masses of molecular ions is simple. One advantage of the ESI technique over MALDI is that ESI can tolerate the salts and detergents found in buffer systems (Aebersold and Goodlett, 2001). An ESI mass spectrometer is normally connected to a high-pressure liquid chromatography (HPLC) with a microbore or capillary C_{18} column for separation of peptides proteolyzed by modified or non-modified Trypsin. If ESI uses only one mass analyzer, it is called ESI-MS. When it employs two mass analyzers and one collision-induced dissociation (CID) device between the two mass analyzers, it is called ESI-MS/MS or a tandem mass spectrometer (Romijn *et al.,* 2003). This methodology has the ability to resolve peptides in a mixture, isolate one species at a time, and dissociate it into amino or carboxy-terminal-containing fragments. Once members of a multi-protein complex have been identified by mass spectrometry, their function is studied by pertinent assays (Honore, 2001).

VIII. Significance of the Dissertation Research

Babies and children are not just small adults. They differ from the adults in many perspectives, such as physical maturity, physiological components, biochemical function, and metabolic capacity (Gilbert, 2000). It is probable that they are susceptible to hazardous chemicals. Susceptibility to chemicals may change with age. Vulnerability often depends on developmental stage (Rice and Barone, 2000). There are critical periods of structural and functional development during both pre-natal and post-natal life and a particular structure or function will be most sensitive to disruption during its critical periods (Weiss, 2000).

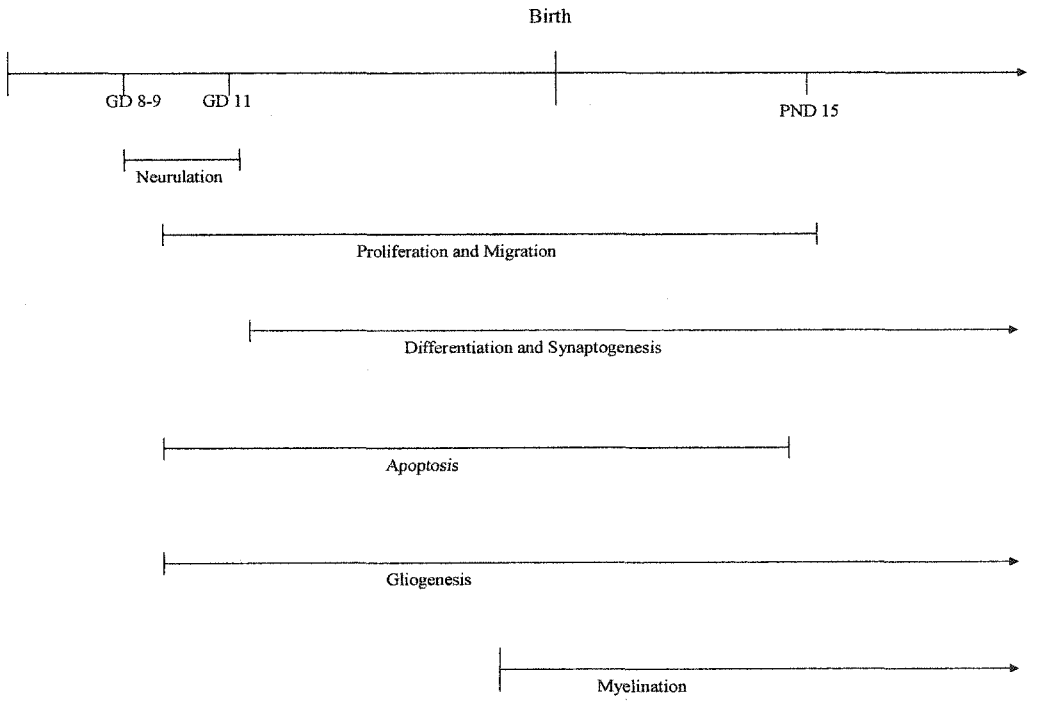

Fig. 1.3. Timetable of developmental processes in nervous systems of rats. GD, gestational day; PND, postnatal day

There are also differences in pharmacokinetics and metabolism between the young and adults. Babies and children may have lower capacity to repair damage from chemical insults than adults (Kimmer and Makris, 2001). Damage may not be evident until a later stage of development. These points firmly suggest why the research in babies and children should be distinct from that in adults.

It is increasingly recognized that humans are generally not exposed to single chemicals. Therefore knowledge of individual chemical toxicity is often inadequate for risk assessment. Our inability to predict whether toxic agents will act in an additive, synergistic, or antagonistic fashion at environmental levels creates huge

problems for human health risk assessment. Many examples exist where the interactions of chemicals with each other or other agents affect human health to a greater extent than would have been predicted based on toxicity of the individual components (Groten, 2000). Chemical mixtures are ubiquitous in the air we breathe, the food we eat, and the water we drink. Chemicals with different modes of action may affect the same organ or tissue and thus interact with each other. Therefore, it is important to develop a rational approach to studying mixtures by combining mechanistic studies with computational modeling strategies and to prioritize chemical mixtures for study based on known human exposures.

Studies of lead (Pb) provided the first paradigm for the concept of subclinical neurotoxicity in children. Pb-induced mental deficits appeared to be permanent, and were associated with an increased risk for violent behavior in adult life. Further studies implicated that low-dose exposure to other classes of chemicals, including MeHg and PCBs resulted in neurodevelopmental abnormalities. These findings demonstrate that the developing nervous system is uniquely vulnerable to the chronic low-dose exposure to multiple chemicals. Despite the potential that babies and children are exposed to multiple classes of neurotoxicants in the environment, risk assessment in this area is still rudimentary. Epidemiological and toxicological studies on developmental neurotoxicities have focused on individual chemical effects. The mechanistic studies failed to cover up changes of chemical effects during critical period in development. These approaches have shortcomings: First, risk assessment for chemical mixtures is based on the additivity of toxic effects of individual chemicals. This is challenged primarily due to the fact that one chemical in the

mixture could affect the toxicities and/ or pharmacokinetics of the other chemicals (Groten, 2000). For example, co-administration with PCB 153 antagonized the induction of cleft palate by TCDD (Morrissey *et al.*, 1992). Second, the developing brain is more sensitive to environmental hazards than the adult brain because there are rapid changes in growth and development during a short time period (Rice and Barone, 2000). Without consideration of the different sensitivities and biological activities of the brain during the developmental period, risk estimations for neurotoxicities during this critical time would be over or under-estimated (Kimmel and Makris, 2001).

All of the above considerations motivate the need to develop a research strategy for combining toxicological assessment with exact tissue dosimetry. In addition, molecular and cellular mechanisms underlying the susceptibility of the developing brain to the exposure of environmental contaminants must be investigated within the context of pharmacokinetic profiles of chemical mixtures during developmental period. While the toxic and biological effects of each agent are well known, little emphasis is placed on studying the interaction of multiple chemicals on normal cell functions. In conclusion, the need to assess effects of chemical mixtures on brain development is of high priority.

The underlying purpose of the study is to investigate the potential whether MeHg and PCBs can induce interactive effects in developing brain using experimental toxicology and computational modeling. To assess interactive effects between MeHg and PCBs in mice, both pharmacokinetic and pharmacodynamic interactions were investigated through experimental methodologies and

computational simulation. The approach integrated tissue dosimetry, PBPK modeling, and proteomics. In subsequent chapters, objectives for each study will be presented, as well experimental understandings and the resulting data. In chapter 2, the study investigating pharmacokinetic interactions between PCB 153 and PCB 126 in non-lactating mice, lactating mice and their pups are described. In chapter 3, PBPK modeling describing the lactational transfer of PCB 153 and its pharmacokinetic interactions with PCB 126 are addressed. In chapter 4, pharmacokinetic interactions between MeHg and PCB congeners for the lactational transfer are described and a PBPK model is developed to aid in mechanistic understanding of these interactive effects. In chapter 5 and 6, pharmacodynamic interactions between MeHg and PCB congeners in developing brain using a proteomics approach is explored. Finally, the research described herein will be summarized and future directions will be discussed improving these studies in the final chapter (chapter 7).

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Table 1.1. Summary of toxic effects of MeHg and PCBs on developing brain

	MeHg Effect	MeHg Mechanism	PCB Effect	PCB Mechanism
Brain Growth, or Neurulation	Mitosis (Choi, 1991) ↓ Cell cycle (Ou, *et al.*, 1997) ↓	Microtubule depolymerization (Hunter and Brown, 2000) Induction of regulatory gene (Ou, *et al.*, 1999),	Cell proliferation in embryo ↓ (Kuchenhoff, *et al.*, 1999)	
Proliferation	Cell proliferation (Miura, *et al.*, 1998) ↓ Cell number (Sager, *et al.*, 1984) ↓	Microtubule depolymerization (Miura, *et al.*, 1998)	Cell proliferation ↓ (Porterfield, 2000) Hippocampul fiber growth ↓ (Pruitt, *et al.*, 1999)	Thyroidal hormone disruption (Porterfield, 2000)
Migration	External granular cell migration in cerebellum ↓ (Kunimoto and Suzuki, 1997)	Apoptosis increase (Kunimoto, 1994)	Actin depolymerization ↓ (Porterfield, 2000)	Thyroidal hormone disruption (Porterfield, 2000)
Differentiation	Limb bud, CNS cell differentiation ↓ (Parran, *et al.*, 2001)	Cell arrest gene ↑ (Ou, *et al.*, 1999) PKC disruption due to –SH interaction (Haykal-Coates, *et al.*, 1998)[a]		
Synaptogenesis			Delayed synaptogenesis (Chou, *et al.*, 1979), Cholinergic fiber ↓	Thyroidal hormone disruption (Sher, *et al.*, 1998)
Gliogenesis	Astrocyte swelling ↑ (Vitarella, *et al.*, 1996)	PLA_2 activation (Ashner, 2000), Activation of the Na+/H+ antiporter (Ashner, *et al.*, 1998)	GFAP ↓, Synaptophysin ↑ (Morse, *et al.*, 1996)	Thyroidal hormone disruption (Sher, *et al.*, 1998)
Apoptosis	Acceleration of normal apoptosis process (Kunimoto, 1994)	Caspase 3-like gene activation (Robertson and Orrenius, 2000)		

Myelination	Myelination ↓ (Vinay, *et al.*, 1990) Cell recognition ↓ (Jacobs, *et al.*, 1986)			
Neurotrophin/ Neurotrans-mitter	Trk-like Rc. ↓ (Barone *et al.*, 1998) BDNF ↓ (Andersson, *et al.*, 1997), mAch Rc. ↓ (Von Berg, *et al.*, 1980) Dopamine Rc. Antagonism (Taylor and DiStefano, 1976) IGF antagonism (Bulleit and Cui, 1998) NGF ↓ and antagonism (Soderstrom and Ebendal, 1995) Glutamergic Rc ↓ (Rajanna, *et al.*, 1997) Dopaminergic Rc ↓ (Cajiano, *et al.*, 1990) GABA uptake ↓ (Kumamoto, *et al.*, 1986) NT release, reuptake ↓ (Sarafian and Verity, 1993)	Calcium signaling perturbation (Marty and Atchison, 1997) Na^+, K^+ ATPase ↓ (Maier and Costa, 1990) Mitochondria disruption (Provan and Miyamoto, 1995) Oxidative stress (Woods and Ellis, 1995)	dopamine ↓ GABA Rc. ↓ Serotonergic cells ↓ Choline acetyl transferase ↓ GABA ↑ neurotransmitter reuptake ↓	Thyroidal hormone disruption (Porterfield, 2000), Calcium signaling disruption (Tilson and Kodavanti, 1998)
Pups and the young			LTP ↓ (Hussain, *et al.*, 2000)	Choli-nergic signaling disruption (Altmann, *et al.*, 2001)

[a]List of abbreviations: PKC, protein kinase C; PLA_2, protein lipase A2; GFAP, glial fibrillary acidic protein; Trk, trophic receptor kinase; BDNF, brain-derived neurotrophin ; IGF, insulin-like growth factor; NGF, neurotrohic growth factor; GABA, gamma aminobutyric acid; NT, neurotrophin

CHAPTER 2

Comparison of Pharmacokinetic Interactions and Physiologically-based Pharmacokinetic Modeling of PCB 153 and PCB 126 in Non-pregnant, Lactating Mice and Suckling Pups

Sun Ku Lee, Ying C. Ou, and Raymond S.H. Yang

ABSTRACT

PCBs are ubiquitous environmental contaminants that can induce neurological defects in infants and children via placental and lactational transfer. To investigate the lactational transfer of PCBs and compare pharmacokinetic interactions among non-pregnant, lactating mice, and suckling pups, quantitative time-course measurements of PCB accumulation in tissues were performed. On PND 1, non-pregnant and lactating C57BL/6 mice were exposed to PCB 153 (2,2', 4,4', 5,5'-hexachlorobiphenyl, 20 mg/kg) alone or a mixture of PCB 153 (20 mg/kg) and PCB 126 (3,3', 4,4', 5-pentachlorobiphenyl, 0.2 mg/kg) by oral gavage. At 1,3,6, and 13 days after treatment, PCB 153 and PCB 126 were determined in non-pregnant and maternal tissues as well as in neonatal tissues by gas chromatography. Co-administration of PCB 153 and PCB 126 increased PCB 153 retention in the liver and decreased PCB 153 accumulation in the fat of non-pregnant mice. Lactational transfer was confirmed to be an efficient elimination mechanism for the lactating

mice, but a major source of exposure in the pups. However, little or no significant pharmacokinetic interactions were observed in lactating mice and suckling pups. To describe pharmacokinetic interactions between PCB 153 and PCB 126, a physiologically-based pharmacokinetic model for PCB 153 disposition was developed. The effects of PCB 126 on the fat content in liver and a diffusion permeation constant in fat were incorporated into the PBPK model. This model successfully describes PCB 153 disposition altered by PCB 126 in non-pregnant mice.

INTRODUCTION

PCBs are ubiquitous environmental contaminants causing a variety of toxicities in humans and animals (ATSDR, 1999). They are detected in numerous and diverse biological compartments including human sera and have become quantitatively the most important organochlorine residues in the environment due to their lipophilic character and their slow metabolism (Carpenter, 1998;Muhlebach *et al.*, 1991). PCBs usually exist in a mixture form, and can comprise up to 209 different congeners (ATSDR, 1999). Although PCBs have been reported to induce a variety of toxic effects including immunologic, teratogenic, reproductive, carcinogenic, and neurological effects in both human and animal studies (Safe, 1989), developing brains are particularly susceptible to PCB exposure (Schantz *et al.*, 1996). PCBs have been reported to induce neurobehavioral deficits in children born at contaminated sites (Carpenter, 1998). Many of the observed defects were related to the nervous system, including abnormalities on behavioral assessment and increased activity, greater incidence of behavioral problems, lower IQ, and impaired visual recognition (Schantz,

1996). These neurological defects are primarily due to the exposure of the fetus through placenta and/ or maternal milk (Buck, 1996; Seegal, 1996).

A major molecular structural factor in the determination of toxic properties and potencies in PCBs is the presence of chlorine atoms on the ortho-positions, influencing the propensity of the molecule to adapt a planar conformation (Safe, 1994). Some non-ortho or mono-ortho chlorine-substituted PCBs have co-planar structures and high affinity for Ah-receptor. Their toxic effects are mediated by the Ah-receptor, and their toxic potencies are determined by the affinity for this receptor. In contrast, the di to tetra ortho-chlorinated PCBs have non-planar structures and no measurable affinity for Ah-receptor. Their mechanisms of action are not completely understood (van der Burght *et al.*, 1999). While both types of PCB congeners have been reported to cause neurodevelopmental deficits following *in utero* and/or lactational exposure (Hussain *et al.*, 2000; Saghir *et al.*, 2000), the mechanisms of action between planar and non-planar PCBs differ significantly (Humphrey *et al.*, 2000) and interactive effects between two types of PCBs have been observed (van Birgelen *et al.*, 1996; van der Plas *et al.*, 1988).

The developing brain is different from the adult brain in both composition and function (Kalil *et al.*, 2000), and the vulnerability of the developing brain to toxicants is critically dependent on timing and duration of exposure. Exposure to environmental toxicants coincident with the ontogeny is more likely to cause toxicities if they interfere with developmental processes within the critical period (Rice and Barone, 2000). Therefore, characterization of the exact pharmacokinetic profiles of environmental toxicants during the critical period of development is

important both for understanding mechanisms of developmental neurotoxicities and in facilitating interpretation of the results from animal studies.

PCB 153 and PCB 126 were selected as a prototype to understand the potentially complex pharmacokinetic interactions associated with PCB mixtures. These congeners are the ones that appear most prevalently both in the environment and human serum (Humphrey *et al.*, 2000). PCB 153 is a representative non-planar congener that appears in environment and mammalian tissues at the highest concentration (Muhlebach *et al.*, 1991). PCB 126 is the most toxic PCB congener with coplanar structure (Safe, 1994) and binds to Ah receptor with highest affinity. Both congeners have been suggested to induce neurobehavioral deficits via gestational and lactational transfer (Hussain *et al.*, 2000). Pharmacokinetic interactions between co-planar PCBs or TCDD and non planar PCBs were previously reported in adult rats and mice (van Birgelen *et al.*, 1996; van der Plas *et al.*, 1988). However, no studies investigating pharmacokinetic interactions between these two congeners during the critical developmental period have been reported despite the potential importance of these interactions. In reality, PCBs are available to human beings as mixtures. Accordingly, the pharmacokinetic information exposed to single congener is not enough to describe pharmacokinetic profiles during developmental period.

The objectives of our study were three-fold. First, we wanted to investigate whether co-exposure to two different PCB congeners with distinct toxic mechanisms of action (i.e., PCB 153 and PCB 126) could change the pharmacokinetic profiles in mice. Second, we wanted to compare pharmacokinetic interactions among non-

pregnant mice, lactating mice, and suckling pups because PCB congeners could be transferred to the fetus and suckling mice from their mothers (Orberg and Ingvast, 1977) and the extent of transfer was greater in lactation than through the placenta (Vodicnik and Lech, 1980). Finally, PBPK modeling, a simulation technique useful for species extrapolation and quantitative risk assessment (Dedrick and Bischoff, 1969), was used as a hypothesis-testing tool for deriving mechanistic insight for the observed pharmacokinetic data.

MATERIALS AND METHODS

Chemicals. PCB 153 and PCB 74 (2, 4, 4', 5 – tetrachlorobiphenyl) were purchased from Ultra Scientific (North Kingtown, RI). PCB 126 was obtained from Accustandard (New Haven, CT). The purities of all congeners used were over 98 %, as confirmed by both vendors. Pentane (HPLC grade), sea sand, and diethylether (> 99% purity) were purchased from VWR Scientific (Denver, CO). Sodium anhydrous sulfate and Florisil® (pesticide residue grade, 60-100 mesh) were purchased from Alltech Associates (Deerfield, IL).

Animals and treatment. C57 BL/6 pregnant female mice were purchased from Harlan Sprague Dawley Laboratory (Indianopolis, IN) and housed individually in the cages at Painter Center in Colorado State University, which is fully accredited by the American Association for Accreditation of Laboratory Animal care (AAALAC). The mice were maintained on a 12-hr light/dark cycle at a constant temperature of 25 °C and humidity of 55%. Diet (certified Teklad NIH-07 rodent diet) and tap water were provided *ad libitum*.

On PND 1, lactating mice were exposed to PCB 153 alone (20 mg/kg bw) or a mixture of PCB 153 (20 mg/kg bw) and PCB 126 (0.2 mg/kg bw) through oral gavage. Corn oil was used as vehicle to dissolve both congeners. The dosing time was between 8:00 and 9:00 a.m. At 1, 3, 6, and 13 days after oral gavage, the lactating mice and their pups were anesthetized using isofluorane or CO_2 gas and then euthanized. The tissues of lactating mice (liver, fat, skin, and brain) and pups (liver, brain, and the remaining carcass excluding GI tract) were collected from each animal. Non-pregnant mice were treated the same way as lactating mice and their tissues were collected at 1, 6, and 13 days after treatment. All samples were frozen with liquid nitrogen and stored at –70 °C until analysis.

Extraction. Liver, fat, and brain samples were weighed (approximately 0.5 g/sample) and then transferred to stainless steel beakers. Sea sand and sodium sulfate anhydrous mixture (5 g/ 10 g) were added to the samples and then ground using a glass stirring rod until granular dry mass was obtained. PCB 74 (200 ng) was added to each of the ground samples as an internal standard. Twenty ml of pentane was added to each sample, which was then boiled on a hot plate with stirring. The boiling temperature was between 30 °C and 40 °C. The extracts were transferred to the filters and collected in glass tubes. For the extraction of the carcass from pups and skin tissues of adult mice, 10 ml of 50% sulfuric acid was added to each sample. After homogenization, 10 ml of pentane was added and vigorously shaken. When the layers separated, the upper layer was transferred to glass tubes. These processes were repeated three times. Collected extracts were concentrated to 2 ml with a nitrogen evaporator.

Clean-Up. Before clean up, the adsorbents (sodium sulfate anhydrous and Florisil®) were layered (the ratio of sodium sulfate to Florisil® is 1 to 2) and packed into a Florisil® glass column (Allen Scientific Glass, Denver, CO) and then the column was rinsed with 100 ml of pentane. The concentrated extract was transferred to the column. The column was eluted with 100 ml of pentane/diethylether mixture (6% diethylether in pentane). The elution rate was adjusted to 20 drops per 10 seconds. Finally, the collected solution was concentrated to 1.0 ml by a nitrogen evaporator.

Gas chromatographic analyses. A HP-5890 Series II Plus gas chromatograph (Hewlett Packard, Wilmington, DE) with ECD detector was used to determine PCB amount. The analysis condition was based on a previous method (Mills *et al.*, 1963). Briefly, DB-5 (crosslinked 5% phenylmethylsilicone, 30 m * 0.32 mm, Agilent Technologies, CA) capillary column was used. The initial temperature was 60 °C and programmed to 200 °C at 30 °C/min for 6 minutes, subsequently to 250 °C at 3 °C/min for 15 minutes. The flow of carrier gas, helium, was 5 ml/min. The make-up gas was pure nitrogen at a flow rate of 70 ml/min. The injector temperature was 225 °C and the detector temperature was 320 °C. The volume of injection was 2 µl per sample.

Samples were quantitated using an internal standard method as previously reported (Storr-Hansen, 1991). Standard solutions for PCB 153 and PCB 126 were prepared in the range from 5 ng/ml to 500 ng/ml. To each solution, PCB 74 (200 ng/ml) was added as an internal standard. Calibration curve was created and fitted using quadratic regression equation.

Statistics. Differences of tissue concentration between samples from various treatment groups and time points were tested for significance by one-way ANOVA, followed by Fisher's multiple comparison test. All analyses were performed with the statistical software, Minitab ($P< 0.01$; Windows version 12.0).

Construction of an interaction PBPK Model. To explain pharmacokinetic interactions between PCB congeners, a PBPK model for the combination effects of PCB 153 and PCB 126 was developed. The previous PBPK model for PCBs (Lutz *et al.*, 1977) was composed of 5-lumped compartments and successfully described individual PCB disposition including PCB 153 using flow-limited transfer. However, this model could not explain the change of PCB 153 disposition affected by PCB 126.

In order to describe pharmacokinetic changes of PCB 153 upon co-exposure to PCB 126, the PBPK model of Lutz *et al* was modified as follows: First, we incorporated time-dependent increase of partition coefficient in the liver. This was supported by several experimental observations. It has been shown that some co-planar PCBs (e.g., PCB 126) and TCDD induces fat accumulation in the liver in a time-dependent manner (Koga *et al.*, 1990). Furthermore, PCB 126 could increase lipid content by 50% in murine liver 7 days after oral exposure (Nobuyuki *et al.*, 1990). In general, the partition coefficient in tissues is related to the n-octanol/ water coefficient, Kow, and profiles for water and lipid contents in each tissue (De Jongh *et al.*, 1997). Since PCB 153 is lipophilic compound, its partition coefficient in tissues is highly proportional to lipid content in tissues. Therefore, the partition coefficient in the liver (PL) was modified to a time-dependent equation, $PL = 10 + \alpha*TIME$ where α is the coefficient for time-dependent increase of partition coefficient and

TIME is the minutes after PCB 153 exposure. Second, the fat compartment was described as diffusion-limited and a diffusion permeation constant (PAFC) from fat-blood to fat tissues was added. It has been suggested that PCB 153 circulates in the body in association with lipoproteins. To be absorbed in fat tissues, PCB 153 was suggested to be dissociated with lipoproteins by lipoprotein lipases (Noren *et al.*, 1999). Because TCDD can inhibit lipoprotein lipase in the fat (Olsen *et al.*, 1998), it is likely that a TCDD-like PCB congener such as PCB 126 can also inhibit lipoprotein lipase, thus decreasing the uptake of PCB 153 in the fat. Both *in vivo* and *in vitro* studies showed that lipoprotein lipase in adipocyte was inhibited as soon as 1 hr after TCDD administration (Olsen *et al.*, 1998). Accordingly, diffusion permeation constant from fat-blood to fat tissues (PAFC) was assumed to decrease by co-exposure to PCB 126 and PCB 153. Third, brain compartment was added as a major target organ because PCBs can induce neurotoxicities in both adult and young mice.

A schematic diagram for this PBPK model is shown in Figure 2.1. Other physiological and biochemical parameters were obtained from a report by Brown *et al.* (1997) and a previous paper (Lutz *et al.*, 1977) and shown in Table 2.1. All PBPK model construction, simulation, and parameter estimations were performed using the Berkeley Madonna software package (version 8.01 for Windows, Kagi Shareware, Berkeley, CA).

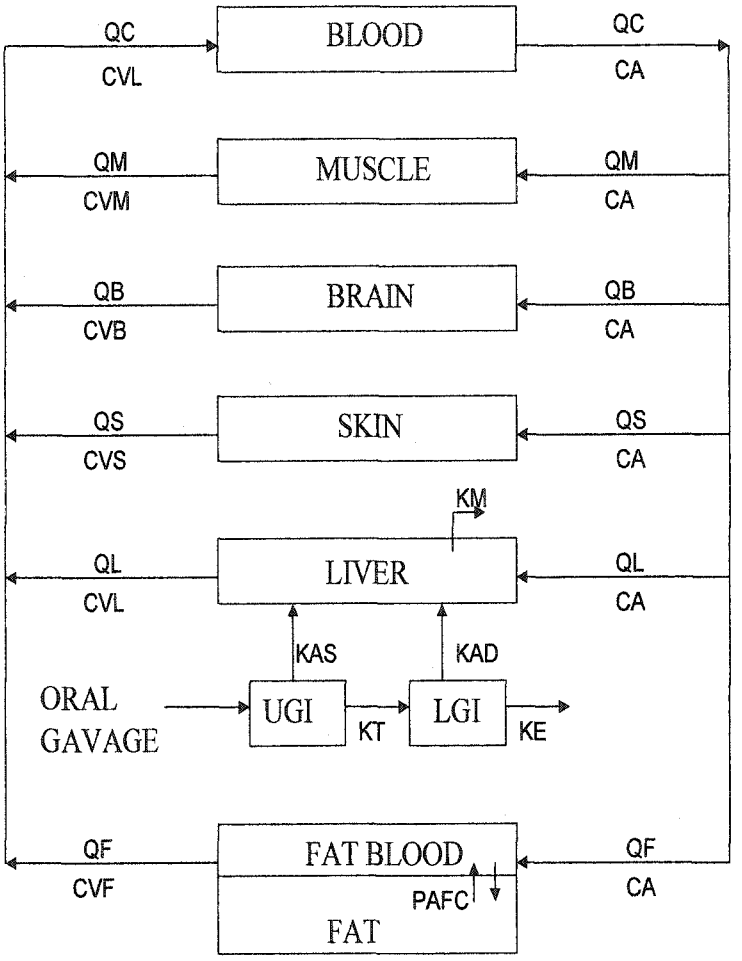

Fig. 2.1. A schematic diagram of the physiologically based pharmacokinetic model used to describe pharmacokinetic interaction on PCB 153 disposition in the non pregnant mice. All compartments except fat are described as flow-limited.

Table 2.1. PBPK Model Parameters

Parameter	Abbreviation	Value	Parameter estimation
	Physiological Parameters		
Body weight (kg)	BW	0.025	Literature[a]
Fraction of brain	BF	0.020	Literature[a]
Fraction of blood	BLF	0.049	Literature[a]
Fraction of fat	FF	0.056	Literature[a]
Fraction of skin	SF	0.208	Literature[a]
Fraction of muscle	MF	See legend[b]	Literature[a]
Fraction of GI tract	GF	0.042	Literature[a]
Fraction of liver	LF	0.080	Literature[a]
Cardiac output (l/hr)	QC	16.5*BW**0.75	Literature[a]
Fraction of blood flow of brain	QBF	0.033	Literature[a]
Fraction of blood flow of fat	QFF	0.07*BW**0.75	Literature[a]
Fraction of blood flow of skin	QSF	0.083	Literature[a]
Fraction of blood flow of muscle	QMF	See legend[c]	Literature[a]
Fraction of blood flow of GI tract	QGF	0.141	Literature[a]
Fraction of blood flow of liver	QLF	0.159	Literature[a]
	Partition Coefficients		
Brain	PB	2.5	Fitted[d]
Fat	PF	300	Literature[e]
Skin	PS	35	Literature[e]
Muscle	PM	5	Literature[e]
Liver	PL	10	Literature[e]
	Biochemical Parameters		
Diffusion permeation constant in fat	PAFC	10	Fitted[d]
Absorption coefficient in upper GI tract	KAS	0.415	Fitted[d]
Absorption coefficient in lower GI tract	KAD	0.077	Fitted[d]
Elimination coefficient in upper GI tract	KT	10	Fitted[d]
Elimination coefficient in lower GI tract	KE	0.073	Fitted[d]
Metabolic clearance (l/hr)	KMET	0.006	Literature[e]

[a]Brown *et al.*, 1997
[b]1-(BF+BLF+FF+SF+GF+LF)
[c]1-(QBF+QFF+QSF+QLF+QGF)
[d]optimized by fitting model output to experimental data
[e]Lutz *et al.*, 1977

RESULTS

Effect of co-exposure with PCB 126 and PCB 153 on body weight gain in growing pups.

PCB 126 has been previously reported to suppress normal weight gain in growing rats (van Birgelen et al., 1994), so the body weights of non-pregnant mice, lactating mice, and suckling pups were monitored after treatment. At the dosing regimen, no differences in body weights were observed between the group exposed to PCB 153 alone and the group co-exposed to PCB 153 and PCB 126 (Data not shown).

Baseline pharmacokinetic interactions of co-exposure with PCB 126 and PCB 153: distribution of PCB 153 in the tissues of non-pregnant mice.

Pharmacokinetic interactions indeed occurred in non-pregnant mice when PCB 153 and PCB 126 were given to them as a mixture. The most significant findings are: (1) time-dependent retention of PCB 153 in the liver when co-exposed to PCB 126; and (2) a reduction in the rate of PCB 153 accumulation in the fat when co-exposed to PCB 126. The details in relation to figures and tables are given below.

Figure 2.2 (A) showed the change of PCB 153 distribution in the liver between the group exposed to PCB 153 alone and the group co-exposed to PCB 153 and PCB 126. At 1 day after treatment, there were no differences in the concentration of PCB 153 between two groups. However, the concentration of PCB 153 in the liver exposed to both congeners was over two times higher than that exposed to PCB 153 alone at 13 days after treatment.

Similar results were shown in the change of PCB 153 distribution in the brain between the group exposed to PCB 153 alone and the group co-exposed to PCB 153 and PCB 126 (Fig. 2.2 (B)). At 13 days post-treatment, the concentration of PCB 153 in the group co-exposed to PCB 153 and PCB 126 seemed to be higher than that in the group exposed to PCB 153 alone, but this difference was not statistically significant.

Co-exposure of PCB 153 and PCB 126 changed the distribution pattern of PCB 153 in the fat. As shown in Figure 2.2 (C), the group exposed to PCB 153 alone showed that the concentration of PCB 153 in fat increased rapidly until reaching plateau (6 days after treatment). The concentration of PCB 153 in the group co-exposed to PCB 153 and PCB 126 increased slowly during that time suggesting a reduction of the rate of uptake.

Skin is the major deposit for PCBs (Vodicnik and Lech, 1980). Figure 2.2 (D) showed the change of PCB 153 distribution in skin between the group exposed to PCB 153 alone and the group co-exposed to PCB 153 and PCB 126. There were no significant differences in PCB 153 concentration between the group exposed to PCB 153 alone and the group co-exposed to PCB 153 and PCB 126.

Postnatal Pharmacokinetics: Comparison of PCB 153 distribution on PND 2 and 14 among non-pregnant, maternal, and pup's tissues.

The pharmacokinetic interactions described above for non-pregnant mice after co-exposure to PCB 153 and PCB 126 did not occur in lactating mice and suckling pups. However, there was strong evidence to confirm earlier studies that lactation is

an efficient mechanism for transfer of PCB 153 to the pups. Consequently, the lactating mice had the lowest PCB 153 concentrations in the liver and brain among three groups of mice and the pups had the highest concentrations in the liver in early lactation period.

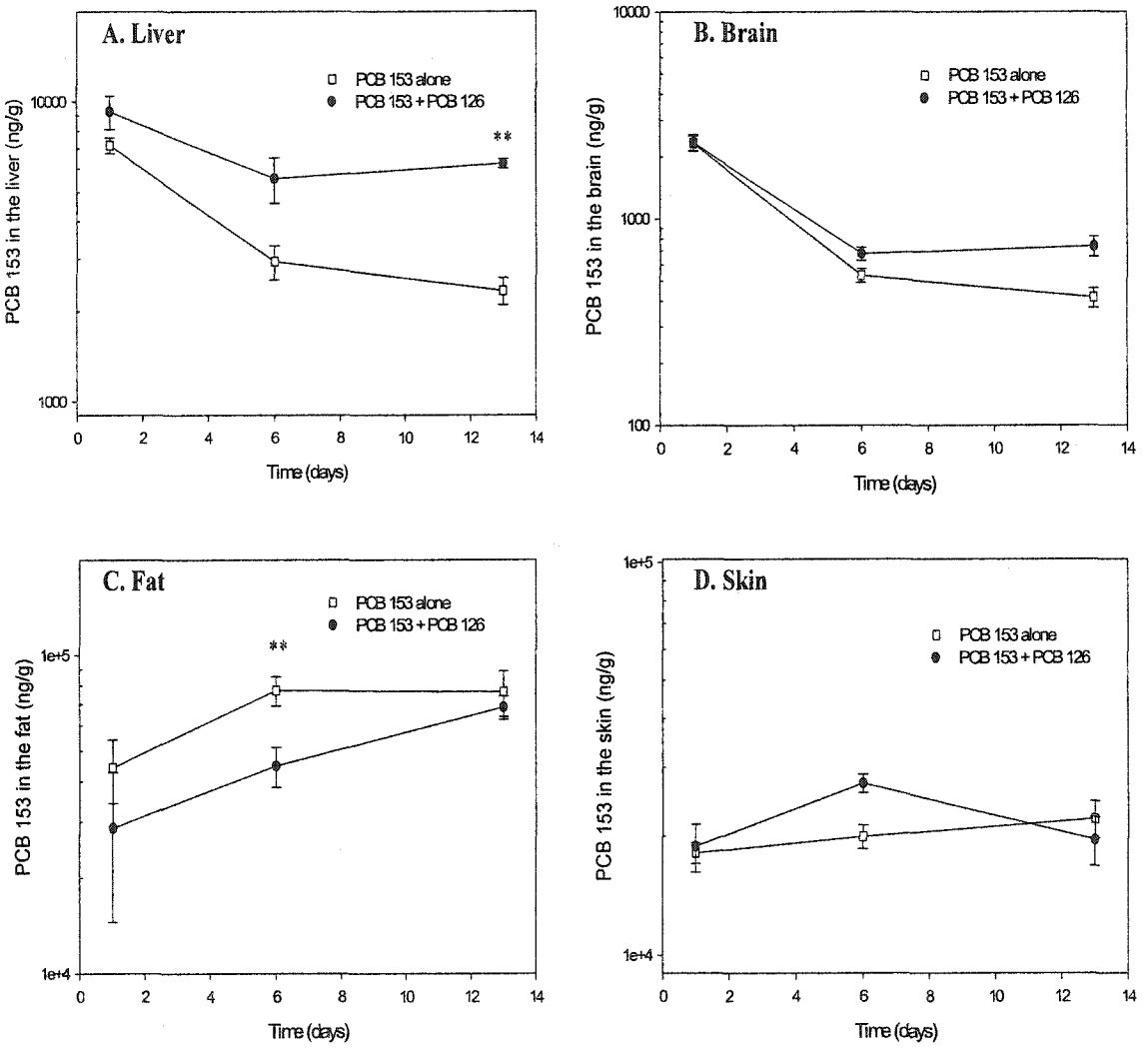

Fig. 2.2. The concentration of PCB 153 in the tissues of nonpregnant mice as a function of time (n=3). **designates statistically significant difference between groups ($p < 0.01$)

We investigated differences in lactational transfer between the group exposed to PCB 153 alone and in combination with PCB 126. Also, we attempted to compare pharmacokinetic interactions among non-pregnant mice, lactating mice, and their pups. The tissue concentrations of PCB 153 in mice of different stages were compared at PND 2 and 14 after oral gavage on PND 1. Figure 2.3. showed the differences of PCB 153 concentration in the liver among non-pregnant, lactating mice, and their pups. Interestingly, the concentrations of PCB 153 in neonatal livers were much higher than non-pregnant and lactating mice on PND 2. However, PCB 153 in neonatal livers was rapidly eliminated; thus, there were no longer any differences in PCB 153 concentrations of livers between non-pregnant mice and suckling pups by PND 14. Co-exposure to PCB 126 increased the retention of PCB 153 in the liver of non-pregnant mice. There were no effects on PCB 153 retention in the liver of lactating mice and their pups. Figure 2.4. showed the concentrations of PCB 153 in the brains of non-pregnant mice, lactating mice and suckling pups. At PND 2, the concentrations of PCB 153 were higher in the brains of non-pregnant mice than those of lactating mice and suckling pups. By PND 14, there was no difference between non-pregnant mice and suckling pups. In contrast, the concentrations of PCB 153 in the brain of lactating mice were significantly lower than those brains of suckling pups and non-pregnant mice at PND 14. Co-exposure to PCB 126 did not change the concentration of PCB 153 in the brains of lactating mice and pups.

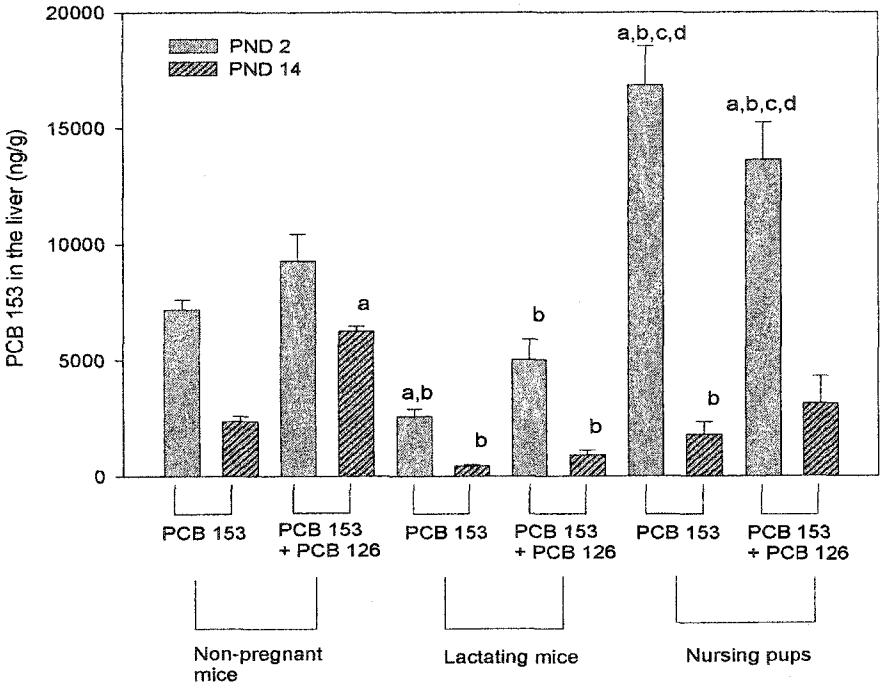

Fig. 2.3. Comparison of concentrations of PCB 153 in the liver of non-pregnant mice, lactating mice, and suckling pups. a: statistically different from non-pregnant mice exposed to PCB 153 only at same date, b: statistically different from non-pregnant mice exposed to PCB 153 + PCB 126 at same date, c: statistically different from lactating mice exposed to PCB 153 only at same date, d: statistically different from lactating mice exposed to PCB 153 + PCB 126 at same date; $p<0.01$).

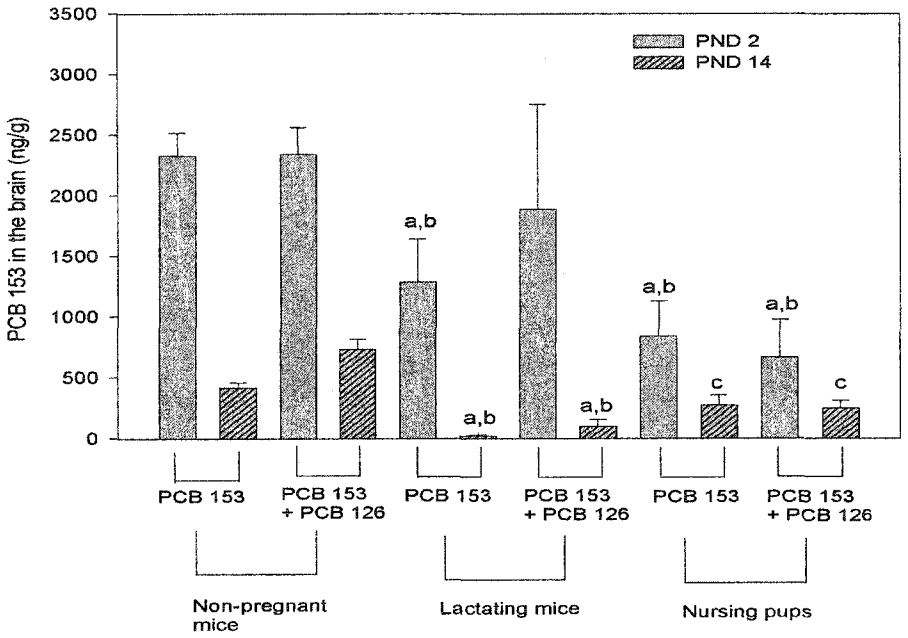

Fig. 2.4. Comparison of concentrations of PCB 153 in the brain of non-pregnant mice, lactating mice, and suckling pups (a: statistically different from non-pregnant mice exposed to PCB 153 only at same date, b: statistically different from non-pregnant mice exposed to PCB 153 + PCB 126 at same date, c: statistically different from lactating mice exposed to PCB 153 only at same date; $p<0.01$).

Figure 2.5. showed the difference of PCB 153 concentration in fat tissues between non-pregnant and lactating mice. In non-pregnant mice, PCB 153 concentration increased at PND 14 from at PND 2. Whereas, PCB 153 did not change or slightly decreased at PD 14 in lactating mice. Co-exposure of PCB 126 did not change the disposition of PCB 153 in fat tissues at PND 14. In both groups, PCB 153 was higher in non-pregnant mice than that in lactating mice at PND 14.

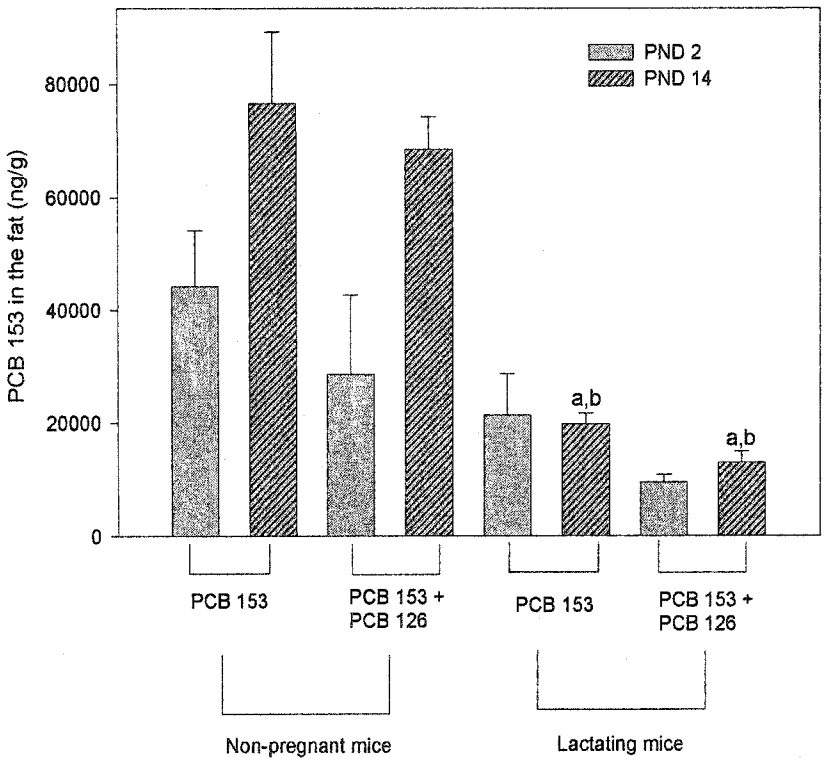

Fig. 2.5. Comparison of concentrations of PCB 153 in the fat of non-pregnant mice and lactating mice (a: statistically different from non-pregnant mice exposed to PCB 153 only at same date; b: statistically different from non-pregnant mice exposed to PCB 153 + PCB 126 at same date, $p<0.01$).

Time-course distribution of PCB 153 in suckling pups with or without co-exposure to PCB 126.

Figure 2.6. (A) showed the change of PCB 153 distribution in pup's liver between the groups exposed to PCB 153 alone and the group co-exposed to PCB 153 and PCB 126. In both groups, the concentration of PCB 153 in neonatal liver decreased over time. There were no significant differences in PCB 153 concentration

at each time point between two groups. This result was different from that of non-pregnant mice, which showed that co-exposure of PCB 126 and PCB 153 increased the retention of PCB 153 in the liver. There were no significant differences of PCB 153 distribution in pup's brain between two groups (Fig. 2.6. (B)). The concentration of PCB 153 in the brain of both groups has decreased after treatment.

Fat tissues in neonatal mice were not significant enough to be reliably collected and analyzed. Therefore, PCB 153 was measured in the pup's carcass without liver, brain, and G.I tract. As shown in Figure 2.6. (C), the concentration of PCB 153 in the carcass increased until 3 days after treatment and then decreased slightly in the group exposed to PCB 153 alone whereas maintained at almost same level in the group co-exposed to PCB 153 and PCB 126. There were no significant differences in PCB concentration at each time point between two groups.

Mechanistic consideration for pharmacokinetic interactions between PCB congeners and PBPK modeling.

The preceding results suggested that co-exposure to PCB 126 increased the retention of PCB 153 in the liver and decreased the absorption of PCB 153 in the fat of non-pregnant mice. These alterations were not observed in those of lactating mice and nursing pups.

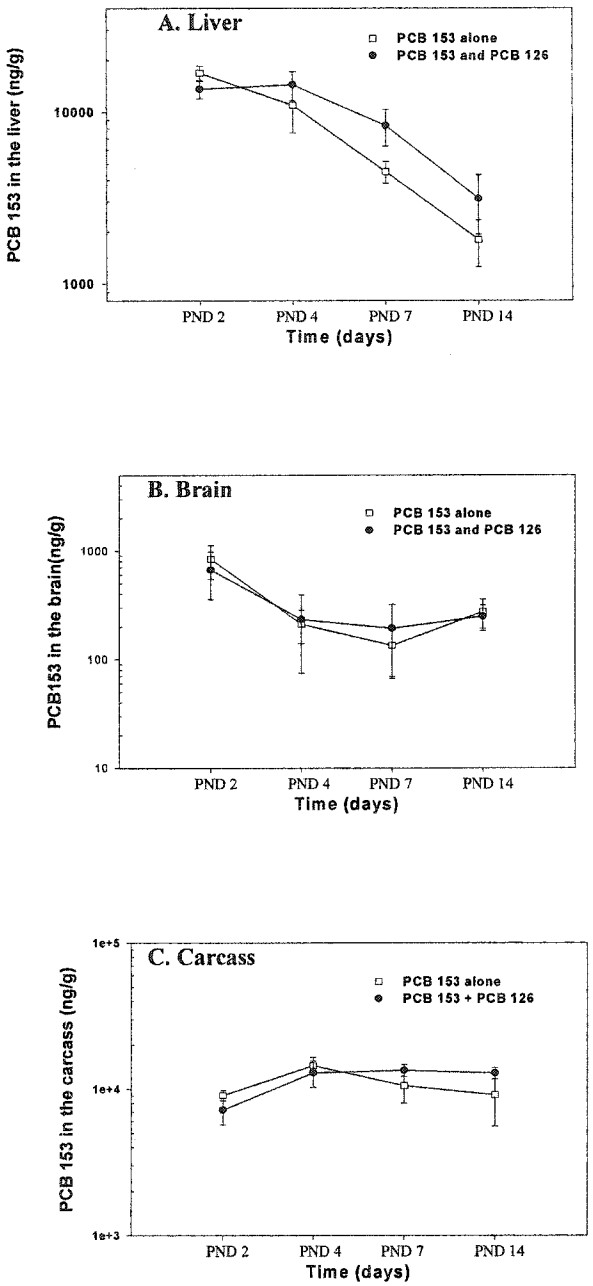

Fig. 2.6. The concentration of PCB 153 in the tissues of suckling pups as a function of time. Values are expressed as the amount of PCB 153 per one gram of tissue. Data points represent mean ± SEM.

As indicated earlier, the mechanistic basis for co-exposure to PCB 126-induced pharmacokinetic interactions were hypothesized to be: (1) time-dependent fat accumulation in the liver; and (2) the decrease of PCB 153 uptake in the fat due to the lack of dissociation of PCB 153:lipoprotein complex through the inhibition of lipoprotein lipase.

In order to address these mechanistic considerations and quantitatively analyze the effects of PCB 126 on tissue distribution of PCB 153, an interaction PBPK model for PCB 153 was developed. Through parameter optimization of two coefficients (α, PAFC), the change of pharmacokinetic profiles of PCB 153 was simulated successfully (Fig. 2.7.). The result of parameter optimization was shown in Table 2.2. Thus, a PBPK model incorporating fat accumulation in the liver and inhibition of lipoprotein lipase activity in the fat adequately described the change of PCB 153 disposition induced by PCB 126.

Table 2.2. Modified PBPK parameters to fit pharmacokinetic changes of PCB 153 affected by PCB 126

Parameter	Abbreviation	Before Modification	After modification
Partition coefficient in liver	PL	10	10 + 0.045*TIME
Diffusion permeation constant in fat	PAFC	10	0.318

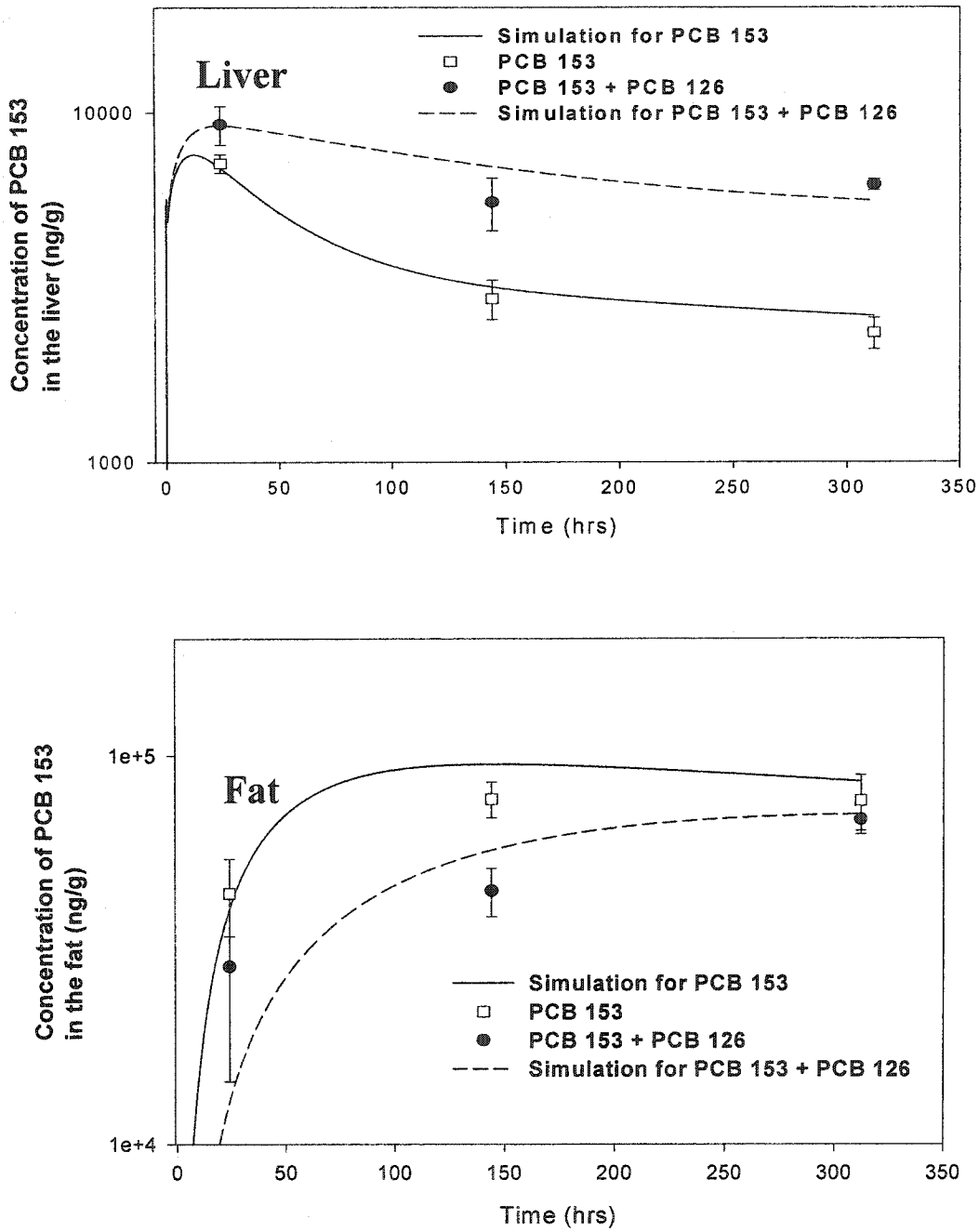

Fig. 2.7. Models simulations (solid and dashed line) of PCB 153 in the tissues of nonpregnant mice.

DISCUSSION

Our most significant results can be summarized as follows: First, co-exposure to PCB 153 and PCB 126 increased the retention of PCB 153 in the liver and decreased the rate of PCB 153 accumulation in the fat of non-pregnant mice. Second, the incorporation of a time-dependent increase of partition coefficient in the liver and the decrease of diffusion permeation constant in the fat into a PBPK model could adequately account for pharmacokinetic changes of PCB 153 in non-pregnant mice. Third, co-exposure to PCB 126 and PCB 153 may not affect the extent of lactational transfer of PCB 153. However, lactational transfer is an efficient elimination mechanism for the lactating mice, but a major source of exposure in the pups. Fourth, interactive effects of PCB 126 on the disposition of PCB 153 are different among non-pregnant mice, lactating mice, and their pups.

The present study demonstrated that co-exposure of C57 BL/6 female mice to a co-planar PCB congener (PCB 126) together with a non-planar congener (PCB 153) affects the redistribution of non-planar congener. In general, PCB 126 is assumed to exert its biological or toxicological actions through Ah-receptor mediated mechanism (Safe, 1984). TCDD, which is also assumed to bind and transduce biological action via interactions with Ah-receptor, was previously shown to increase the retention of PCB 153 in B6C3F1 mice (van Birgelen *et al.*, 1996). Therefore, the effect of PCB 126 on the increase of PCB 153 retention may be through an Ah-receptor mediated process. TCDD is also able to induce fat accumulation in the liver (van Birgelen *et al.*, 1996). Accordingly, the increase of fat content in the liver may increase PCB 153 retention due to the highly lipophilic properties of PCB 153 which confers the

highest affinity for fat among PCB congeners. Recently it was also reported that PCB 126 could reduce essential fatty acid and thus resulted in the increase of lipid content in the liver (Matsusue *et al.*, 1999). These studies suggest that TCDD and dioxin-like PCB congeners can increase the retention of non-planar lipophilic congeners in the liver through the increase of lipid content in liver. In our studies, we adopted this mechanism because it is consistent with our experimental results.

Since PCBs have high lipophilicity and low metabolism, the adipose tissue is the major storage site of most PCB congeners except dioxin-like congeners (Muhlebach *et al.*, 1991). However, our results showed that there was a decreased uptake of PCB 153 into the fat of non-pregnant mice co-exposed to PCB 153 and PCB 126. One possible mechanism is the decrease of lipoprotein lipase activity in adipose tissue by TCDD or co-planar PCBs. In support of this possibility, Olsen *et al.* (1998) showed that TCDD induced a statistically significant time- and dose-dependent decrease in lipoprotein lipase activity in preadipocyte cell line and suggested that different PCB and dioxin congeners could reduce lipoprotein lipase activity through the Ah receptor activation. Lipoprotein lipase on the membrane of adipocyte hydrolyzes lipoproteins into free fatty acids and proteins. PCB 153 could bind to lipoproteins in the liver and be transported to other tissues via lipoproteins (Noren *et al.*, 1999). In fat tissues, PCB 153 is dissociated from lipoproteins by lipoprotein lipase and released into the fat (Gallenberg and Vodicnik, 1987). Therefore, the inhibition of lipoprotein lipase activity by co-planar PCB in the fat could result in the decrease of PCB 153 uptake by adipose tissues.

Through the incorporation of effects of lipid accumulation in the liver and inhibition of lipoprotein lipase in the fat, the observed pharmacokinetic changes of PCB 153 in non-pregnant mice could be successfully simulated using PBPK modeling. It should be noted that the interaction PBPK model in this paper is still preliminary in nature. The time-dependent change of partition coefficient for the liver compartment provided an adequate fit to the data collected during the experimental period of 14 days. However, if the experimental period is very long, the changes of partition coefficient in the liver may become unrealistically large. Of course, such long term accumulation of fat must be verified with experimental results if subsequent PBPK model refinement is to be attempted. Further, as the effects of PCB 126 may be related to receptor binding (i.e., Ah receptor), the concentration of PCB 126 may become an important factor regarding the fat accumulation in the liver in multiple-dose level studies. Refinement of this interaction PBPK model is in progress in our laboratory.

PBPK models have been increasingly useful mechanistic tools to describe and estimate the disposition of chemicals in biological systems (Dedrick and Bischoff, 1969). The purpose for the construction of PBPK model in this study is to quantitatively account for observed interactive effects between PCB 153 and PCB 126 at different stages of development. As in any modeling work in toxicology, the ultimate goal is to predict target tissue dosimetry following the establishment of a validated model. In this regard, the PBPK model for lactational transfer of PCB 153 is now being developed in our laboratory to simulate pharmacokinetic differences between lactating mice and non-pregnant mice.

It was previously reported that non planar PCBs could be readily transferred to offspring (Buck, 1996). Our results support these observations. Beginning in pregnancy, the synthesis of VLDL (very low density lipoprotein) in pregnant mice increases. VLDL is the major lipid form to be hydrolyzed by lipoprotein lipase in the mammary gland to supply free fatty acids for milk triacylglycerol production. It was suggested that the increase of plasma VLDL concentration was responsible for the increased fraction of PCB 153 that was bound to VLDL during pregnancy and lactation (Gallenberg and Vodicnik, 1987). This could be assumed to be the major mechanism of lactational transfer of PCB 153 (Griffin *et al.*, 1991). Because co-exposure of PCB 126 did not affect the lactational transfer of PCB 153, it seems likely that PCB 126 may not influence the function of VLDL both in pregnant mice and lactating pups. However, the function of VLDL in nursing pups has not been investigated. Therefore, further studies on VLDL function in nursing pups may reveal the possible mechanisms on lactational transfer of PCBs.

Contrary to the non-pregnant mice, pharmacokinetic interactions between PCB 153 and PCB 126 were not evident in lactating mice and nursing pups. Pregnancy could induce many physiological changes in the mice. In particular, the changes in the profile of plasma lipids and lipoproteins with late pregnancy could affect the disposition of PCB 153 significantly because PCB 153 is likely transported via lipoproteins with complex interactions (Spindler-Vomachka and Vodicnik, 1984). According to a previous study (Spindler-Vomachka and Vodicnik, 1984), the association profiles of PCB 153 with lipoproteins were changed during pregnancy and lactation. It is not known which lipoprotein is a significant determinant of PCB

153 distribution in suckling pups. Perhaps, these physiological changes related to pregnancy can obscure the effects induced by PCB 126 as shown in non-pregnant mice. To understand the different mechanisms underlying pharmacokinetic interactions in lactating mice and nursing pups, further studies are needed for the effects of PCB 126 on the changes of lipoproteins during pregnancy and lactation period.

Finally, our results can be useful in estimating pharmacokinetic profiles of PCBs transferred by lactation. Historically, neurotoxicological and pharmacokinetic studies of PCBs on developing animals focused on single congeners or commercial mixtures (ATSDR, 1999). In single congener studies, interactive effects between congeners were not considered. In commercial mixture studies, the composition of congeners (e.g. Arochlor 1260) was not the same as that in the environment (Tilson and Kodavanti, 1998). Therefore, the information on chemical and biological interactions between PCB congeners should be incorporated into experimental designs to better understand toxicological responses of PCB mixtures.

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CHAPTER 3

A Physiologically-based Pharmacokinetic Model for Lactational Transfer of PCB 153 with or without co-exposure of PCB 126 in Mice

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ABSTRACT

We present a PBPK model to describe the pharmacokinetics of PCB153 and interaction of PCB 153 and PCB 126 in lactating mice and suckling pups. This model incorporated physiological changes on the volume and blood flow into mammary tissues, and mechanistic changes on the movement of PCB153 from adipose tissue to the mammary gland. We further extended this model to investigate the pharmacokinetic changes of PCB153 upon co-exposure with PCB126 during the lactational period. In non-pregnant mice, parameter changes in fat and liver compartment of the model were required to describe the time-course changes of PCB153 disposition in the tissues upon co-exposure with PCB126. Incorporation of these parameter changes in a lactational model also described better PCB 153 disposition in lactating dams upon co-exposure with PCB126. These results were consistent with earlier experimental findings showing increase of fat amount in the liver and inhibition of lipoprotein lipase in the adipose tissue. Our model suggested that these changes induced by PCB 126 could be a potential mechanism for the effects of PCB126 on the disposition of PCB153. Our model will provide a useful

mechanistic tool to describe and estimate the disposition of PCBs in diverse experimental designs including investigation of PCB effects during developmental period and to facilitate the subsequent interpretation of experimental data.

INTRODUCTION

Basic biological and toxicological information were described in Chapter 2. Briefly, the toxicological impact of PCB exposure has been known after several episodes of accidental exposure in Japan and Taiwan (Kuratsune et al., 1971; Hsu et al., 1985). Several epidemiological studies documented that low-level exposure to PCBs is associated with neurodevelopmental deficits in humans (Jacobson et al., 1990; Huisman et al., 1995). In the United States, a cohort study from Michigan found an association between poor visual recognition memory and prenatal PCB exposure due to maternal fish consumption (Jacobson et al., 1990). Developmental exposure of laboratory animals to PCBs resulted in effects ranging from alterations in sensory and cognitive function, persistent increases in motor activity, to behavioral changes (Tilson et al., 1990). At the cell and molecular levels, developmental exposure to PCB mixtures is reported to disrupt thyroid hormone homeostasis and alter brain dopamine levels (Goldey et al., 1995; Seegal et al., 1997).

Interactive effects among PCB congeners and related compounds have been observed on various biological endpoints. For example, using hepatic cytochrome P450 (CYP) induction, hepatic tumor promotion, and immunotoxicity as endpoints, non-planar PCBs was shown to enhance or suppress the biochemical and toxic response elicited by TCDD in rodents (Van Birgelen et al., 1996; Smialowicz et al.,

1997; Wolfle, 1997). Non-additive interactions between PCB 126 and PCB 153 as promoters of hepatic preneoplastic lesions were observed in rats (Haag-Gronlund et al., 1998). The interactions of PCBs on developmental or CNS toxicity have also been shown. Neonatal exposure by lactational transfer of PCB 77 and PCB 153 showed synergism on induction of hepatic CYP4A1 (Borlakoglu et al., 1992). In addition, co-administration of PCB 153 with TCDD resulted in a 10-fold increase in the incidence of cleft palate induced by TCDD treatment alone (Birnbaum et al., 1985).

Lactational transfer represents the primary route for PCB exposure to the developing organism (Buck, 1996). It has been shown that less than 5% of PCB153 is transferred during gestation but the majority of maternal PCB 153 was transferred to developing pups during lactation (Vodicnik and Lech, 1980). In addition, we showed that co-exposure of PCB 153 and PCB 126 increased PCB 153 level in the liver and decreased PCB 153 accumulation in the fat in non-pregnant mice (Lee et al., 2002). Considering high toxic potentials of PCBs in developing organisms, few studies have been done to investigate pharmacokinetics and pharmacokinetic interactions of PCBs focusing on lactational transfer. Ideally, mechanistic studies regarding transfer mechanisms on lactational transfer of PCBs should be combined with pharmacokinetic studies to better understand the target dose of PCB153 and its interaction with PCB 126 during critical stages of development. Therefore, we developed a PBPK model for lactational transfer of PCB153 including pharmacokinetic interactions between PCB 153 and PCB 126. We incorporated physiological changes during pregnancy and mechanistic considerations such as

potential effects of PCB126 on lipoprotein lipase in adipose tissue or lipid composition in liver tissue. The approach demonstrated here would also be broadly applicable to the study of the increasing number of environmental toxicants or drugs during perinatal development.

METHODS

Chemicals. PCB 153 and PCB 74 were purchased from Ultra Scientific (North Kingtown, RI). PCB 126 was obtained from Accustandard (New Haven, CT). The purities of all congeners used were over 98 %, which was confirmed by both vendors. Pentane (HPLC grade), sea sand, and diethylether (> 99% purity) were purchased from VWR Scientific (Denver, CO). Sodium anhydrous sulfate and Florisil® (pesticide residue grade, 60-100 mesh) were purchased from Alltech Associates (Deerfield, IL).

Animals and Treatment. All animal care and treatment were same descrbed in Chapter 2. On PND 1, lactating mice were exposed to PCB 153 alone (20 mg/kg bw) or a mixture of PCB 153 (20 mg/kg bw) and PCB 126 (0.2 mg/kg bw) through oral gavage. Corn oil was used as vehicle to dissolve both congeners. The dosing time was between 8:00 and 9:00 a.m. At 1, 3, 6, and 13 days after oral gavage, the lactating mice and their pups were anesthetized using isofluorane or CO_2 gas and then euthanized. The tissues of lactating mice (liver, fat, skin, and brain) and pups (liver, brain, and the remaining carcass excluding GI tract) were collected from each animal. Non-pregnant mice were treated the same way as lactating mice and their tissues were

collected at 1, 6, and 13 days after treatment. All samples were frozen with liquid nitrogen and stored at –70 °C until analysis.

The concentration of PCB 153 or PCB 126 were determined using a HP-5890 Series II Plus gas chromatography with a ECD detector (Hewlett Packard, Wilmington, DE) as previously desribed (Lee et al., 2002).

PBPK Model Development. As there is insufficient data to accurately estimate a number of parameters (oral absorption constants, partition coefficients to mammary gland and brain compartment, etc), we took a stepwise approach to obtain these parameters as described below. Our modeling approach began with a comprehensive description of pharmacokinetic events in non-pregnant mice. The assumption employed was that once the non-pregnant model was parameterized, developing a companion model for lactating animals would only need to take into account physiological changes associated with lactation. We then developed a PBPK model for describing pharmacokinetics of PCB 153 in lactating mice and suckling pups. Finally a PBPK model for lactational transfer of PCB 153 to pups with or without co-exposure of PCB 126 in mice was constructed. Each of these steps is described in detail below.

(1) A PBPK model for describing PK of PCB 153 in non-pregnant mice.

Model compartment. A previous PBPK model by Lutz et al (Lutz et al., 1977; Lutz et al., 1984) was successfully used to describe the disposition of several individual PCB congeners, including PCB 153, via the intravenous exposure route. The model described flow-limited transfer of PCBs in fat, skin, muscle and liver tissue compartments. We expanded their model structure by changing the exposure

route to oral gavage, and included additional compartments, including brain and mammary tissues (Figure 3.1). The brain compartment was added as a major target organ because PCBs can induce neurotoxicity in both adult and young mice. Percentage of urine and fecal excretion was also verified with literature data. While the model of Lutz et al (Lutz et al., 1977; Lutz et al., 1984) used flow-limited uptake in all tissue compartments, others have used diffusion-limited uptake of PCB or PCB-like compounds (Parham et al., 1997; Wang et al., 1997). Flow-limited transport is the case where the membrane transfer resistance is negligible compared to the resistance of convection. For large molecules, membrane-limitations are more significant, thus leading to diffusion-limited transfer. A flow-limited model cannot fit the data of a membrane-limited case, since adjusting tissue-blood distribution ratio cannot change the slope of the tissue distribution curve at the early time points. In present work, we evaluated tissue uptake of PCB153 as both a flow-limited process and a diffusion-limited process and adopted the mechanism showing better simulation.

Mathematical expression of the model. The mathematical expressions of the model which described the mass balance on different compartments are formulated as outlined in Ramsey and Andersen (Ramsey and Andersen, 1984). Equation (1) represents a tissue mass balance equation for a flow-limited transport process.

$$dAi/dt = Qi\,(C_B - C_{Vi}) \qquad (1)$$

Where Ai is amount in ith tissue; Qi is blood flow to ith tissue. C_B is arterial blood concentration; C_{vi} is venous blood concentration leaving ith tissue. $C_{Vi} = Ci / Pi$, where Pi is tissue i/blood partition coefficient, Ci is concentration in ith tissue.

$C_i = Ai / V_i$; where Vi is the volume of ith tissue.

Equation (2)-(3) are tissue mass balance equations for a diffusion-limited transport process.

$$dA_{iB}/dt = Q_i^* (C_B - C_{Vi}) + P_{Ai}^* ((C_i/P_i) - C_{Vi}) \quad (2)$$

$$dA_i/dt = P_{Ai}^* (C_{Vi} - (C_i/P_i)) \quad (3)$$

Where A_{iB} is amount in the blood compartment of ith tissue; Qi is blood flow to ith tissue; P_{Ai} is the diffusion permeation constant of ith tissue. For the liver compartment, additional terms were added to describe a first-order oral absorption of PCB 153 ($K_{AS}^* A_{ST}$, where K_{AS} is the first order absorption rate constant from the stomach and duoedeom lumen to the systemic circulation. A_{ST} is the amount in the stomach and duoedeom lumen) and to account for a first-order metabolism of PCB 153 ($K_{FC}^* C_{VL}$ where KFC is the first order metabolism constant in the liver).

Parameter values. Physiological constants such as blood flow and tissue volume (Table 3.1) were taken directly from published literature (Brown et al., 1997). Tissue partition coefficients of those other than brain and mammary tissue compartments of PCB153 were obtained from the publication of Lutz et al (Lutz et al., 1977; Lutz et al., 1984). Partition coefficients for mammary gland and brain tissues were estimated based on model fitting of data, which were verified against with the calculation from their respective *n*-octanol/water partition coefficients, Kow, according to a previously published procedure (Poulin and Krishnan, 1995a; 1995b). Our modeling exercise began with a verification process that the modified PBPK model with additional parameters (e.g., in brain compartment) were still able to describe the data of a previous publication (Tuey et al, 1980), which were collected following a single IV dose in mice. Metabolism and elimination constants describing fecal and urine

excretion was estimated based on fitting of the model to the data previously published (Tuey et al, 1980). The other parameters including oral absorption of PCB153 and diffusion permeation constants were estimated based on fitting of the model to an experimental data set (Lee et al., 2002).

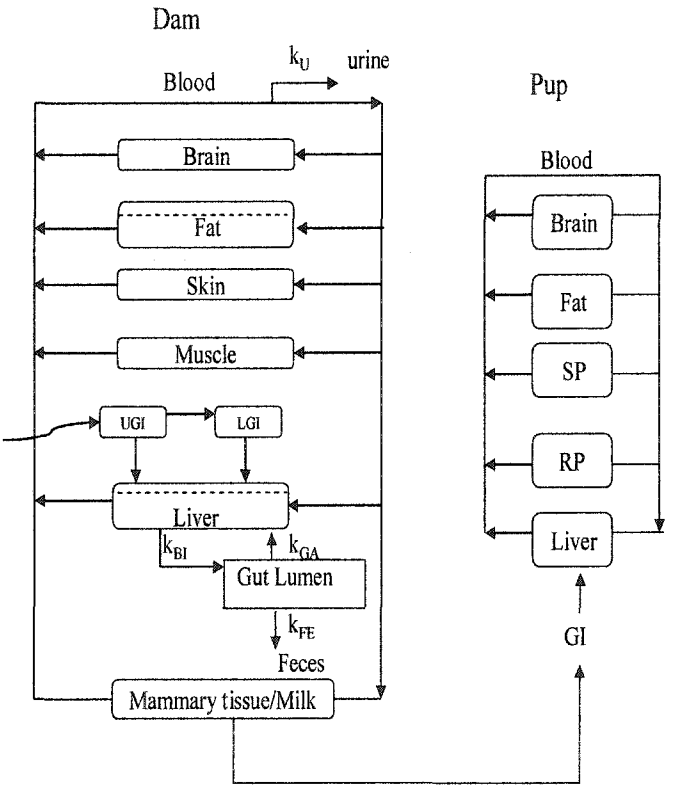

Fig. 3.1. A schematic diagram describing the lactational transfer of PCB 153 in mice.

(2) A PBPK model for describing PK of PCB153 in lactating female mice and suckling pups.

Model compartment. For describing the pharmacokinetics of PCB 153 in lactating dams and suckling pups, additional mammary tissue/milk and pup compartments were added onto the non-pregnant model.

Mathematical expression of the model. Modeling of kinetics in mammary tissue and milk transfer was described similar to the approach by Fisher et al (Fisher et al., 1990). In the mammary tissue, PCB 153 was considered to be flow-limited with respect to maternal blood flow perfusing the tissue. This consideration is based on previous published models, which all assumed flow-limited uptake for mammary tissues. The model with flow-limited uptake in mammary tissue was tested to see whether it was sufficient for the description of data. To model dynamic movement of PCB153 across the mammary tissue from the blood supply into the milk compartment, we evaluated two possible kinetic behaviors. In one approach, we assumed a diffusion process of PCB153 from mammary tissue to milk compartment, with subsequent transfer of PCB153 via milk to pups (with an assumption that milk suckling rate equals milk production rate). In the other approach, we assumed that the maternal blood supply in mammary tissue is in intimate contact with the milk as previously described (Fisher et al, 1990). When the later assumption was used, the mammary tissue and milk compartment is considered as one unit. After evaluating both approaches, we adopted second approach because that assumption described better the tissue concentration data of dams and pups from previous lieteratures

(Vodicnik and Lech, 1980; Lee et al., 2002). The rate change in the amount of PCB153 in the milk compartment of lactating dams comprises two terms; one related to the maternal blood circulation and partitioning to the milk and the other to pup suckling. The equation for the mammary tissue/milk is

$$dA_{MA}/dt = Q_{MA}* (C_B - C_{VMA}) - dAsuck/dt \quad (4)$$

Where A_{MA} is the amount in mammary/milk compartment; Q_{MA} is blood flow to mammary/milk; C_B is arterial blood concentration. $C_{VMA} = C_{MA}/P_{MA}$, where P_{MA} is mammary/milk tissue/blood partition coefficient. C_{VMA} is venous blood concentration leaving mammary/milk tissue. C_{MA} is concentration in mammary/milk tissue. The second term (dAsuck /dt) is identical to the pup litter suckling rate.

$$dAsuck/dt = C_{MA}*K_{MILK} \quad (5)$$

Where K_{MILK} is the milk transfer rate to the pups and was assumed to equal the milk production rate.

The amount of PCB153 transferred to the pup's liver is modeled via equation (6):

$$dA_{LP}/dt = Q_{LP}*(C_{BP}-C_{VLP}) + C_{MILK} * K_{MILK} \quad (6)$$

Where A_{LP} is the amount in pup's liver; Q_{LP} is blood flow to pup's liver; C_{Bp} is aterial blood concentration in the pups. C_{VLP} is venous blood concentration leaving pup's liver.

Modeling approaches/parameter values. The non-pregnant model was modified to incorporate critical time-dependent physiological changes during lactation (Table 3.1) for the description of the lactating dams and suckling pups. The physiological changes associated with lactation were essentially employed by Fisher et al (Fisher et al., 1990), which describes mathematically the dynamic changes in organ/tissue

volume or blood flow rates that occur during lactation. Allometric scaling was used to develop the mouse-specific model here. Mouse growth curve data from the present study was incorporated into the model by linear interpolation using the GRAPH Function in the Berkeley Madonna simulation program. All tissues were assumed to have same density. The growth of fetuses was described according to measurements collected in the present study. The mean fetal body weight is 0.0018, 0.0027, 0.0044, 0.0072 kg on PND 1, 3, 6, and 13. In addition to those physiological parameter changes, many literatures suggested that other physiological changes associated with lactation required the modification of parameter values. These include lipid mobilization during the onset and maintenance of lactation, where an increase in mammary lipoprotein lipase is mirrored by a decline in the lipoprotein lipase level in adipose tissues, and the liver (Spindler-Vomachka and Vodicnik, 1984). These changes play an important role in the fate of circulating triglycerides, which are diverted from uptake by adipose tissue to uptake by the mammary gland for milk synthesis. The need to test these changes in our PBPK model development was further exemplified by the observations that a high proportion of PCB153 in circulation is bound to lipoprotein such as LDL and VLDL (Spindler-Vomachka and Vodicnik, 1984). The uptake of PCB153 into tissues such as liver, fat and mammary tissues could well be governed by dynamic changes in lipid composition in these tissues or lipoprotein lipase activity. We therefore also attempted to evaluate the parameters related to these changes such as kinetics constants in mammary/milk, fat and liver compartment in the model to improve model description of data in lactating dams.

(3) A PBPK model for describing lactational transfer of PCB153 with or without PCB126 in mice.

Modeling approaches/parameter values. We next examined whether the same PBPK model for PCB153 described above could be used to describe the PK of PCB153 in lactating female mice with or without co-exposure of PCB126. With PCB126, some of notable changes in the disposition of PCB153 were previously described (Lee et al., 2002), including higher concentration in the liver, with lower concentration in the fat. Possible mechanistic bases for this interaction were discussed in Chapter 2. Parameterization was followed by a previous model (Lee et al., 2002).

Simulation software and sensitivity analysis. All model construction, simulation, and parameter estimations were performed using the Berkeley Madonna software package (version 8.01 for Windows, Kagi Shareware, Berkeley, CA).

Statistical Analysis. Differences of tissue concentration between samples from various treatment groups and time points were tested for significance by two-way ANOVA, followed by Fisher's multiple comparison test. All analyses were performed with the statistical software, Minitab ($P < 0.01$; Windows version 12.0).

Table 3.1. Physiological constants used in the PBPK model for non- lactating mice, lactating dams and the nursing pups.

	Non-pregnant Mice	Lactating Dam	Pups
		Body weight (kg)	
Body weight (BW)	0.025	0.027-0.032	0.0018-0.0072
		Percentage of body weight (%)	
Blood (VBC)	0.09	0.09	0.09
Gut (VGC)	0.075	0.075	
Liver (VLC)	0.08	0.04	0.04
Fat (VFC)	0.049	0.12 -0.09	0.005-0.08
Fat Blood (VFCB)	0.03	0.05	
Muscle (VMC)	0.91-VFC-VLC-VBC-VSC-VGC-VBRC	0.91-VFC-VLC-VBC-VSC-VGC-VBRC-VMAC	
Skin (VSC)	0.16	0.16	
Brain (VBRC)	0.03	0.03	0.03
Mammary tissue (VMAC)	--	0.044-0.096	--
Slowly perfused (VSPPC)			0.743
Rapidly perfused (VRPPC)			1-VBC-VFC-VBRC-VSPPC
		Flow (liters/hr)	
Cardiac output (QC)	$16.5*BW^{0.75}$	$(14.0\text{-}21.0)*BW^{0.75}$	$18*BW^{0.75}$
		Percentage of cardiac output (%)	
Liver (QL)	0.25	0.25	0.25
Fat (QF)	0.07	0.07	0.09
Muscle (QM)	1-QC-QF-QL-QBR-QS	1-QC-QF-QL-QBR-QS	
Skin (QS)	0.093	0.093	
Brain (QB)	0.025	0.025	0.025
Mammary tissue (QM)	--	0.09-0.15	--
Slowly perfused (QSPPC)			0.2
Rapidly perfused(QRPPC)			1-QL-QF-QB-QSPPC

Table 3.2. Kinetic constants for modeling concentration profile of PCB153 with or without co-exposure of PCB 126 in non-pregnant mice in the lactating mouse and nursing pup.

		Naïve Dam	Naïve Dam (+PCB126)	Lactating Dam	Lactating Dam (+PCB126)	Pups	Pups (+PCB126)
Tissue:blood partition coeffecients							
Liver	PL	10[a]	10+time*0.05[b]	10	10+time* 0.09[b]	25	25
Fat	PF	300	300	300	300	300	300
Skin	PS	35	35	35	35	2.5	2.5
Muscle	PM	12	12	12	12	10	10
Brain	PBR	3	3	3	3	3	3
Mammary	PMA	--	--	80	80		
Pharmacokinetic constants							
Fat diffusion coefficient	PAFC (L/hr)	0.09	0.03	0.09	0.04		
Oral absorption coefficient	KST (/hr)	0.08	0.08				
Linear metabolism rate constant	KFC	0.000 58	0.00058	0.000580	0.00058		
Suckling Rate (milk production rate)	K_{milk}	--	--	0.00009	0.00009		
Elimination rate from pup's liver	K_{elimP}	--	--	0.00001	0.00001		
Biliary clearance	K_{BI} (L/hr)	4.46	4.46	4.46	4.46		
Gut reabsorption	K_{GA}	0.016	0.016	0.016	0.016		
Fecal transport	K_{FE}	0.08	0.08	0.08	0.08		
Kidney clearance	K_U	1.09	1.09	1.09	1.09		

Use subscript to mark the reference sources.

[a] from Tuey et al, 1980.

[b] Time is between 0 and 350 minutes. Final possible PL values are between 10 and 41.5.

RESULTS

(1) A PBPK model for describing PK of PCB 153 in non-pregnant mice. As presented earlier in method section, we first developed a modified PBPK model to describe time-concentration profile following exposure of PCB153 in non-pregnant female mice. A summary of parameters used in the model is shown in Table I and II. This modified PBPK model was able to describe the data following a single IV dose of 0.6 mg/kg PCB153 from the study of Tuey et al (1980). As shown in Figure 2a, the model-predicted liver, fat, blood and skin tissue concentrations closely correspond to experimental data. The model predictions also closely approximate the percentage of urine and fecal excretion changes during the study period. Unlike some PCB congeners, PCB153 is not well metabolized. By the end of 7 days, the total accumulative excretion was less than 10 % in feces and less than 5 % in urine (Figure 2b). By the end of 14 days, the total accumulative excretion via feces was about 20 % (Figure 3.2b).

The same modified PBPK model was also used to describe time-concentration data following a single oral dose of 20 mg/kg PCB153. The experimental data set was published elsewhere (Lee et al., 2002). According to simulation results, the predicted peak concentration of PCB153 in liver, skin and brain is achieved during the first day after the dosing, followed by a gradual decline thereafter. However, in the fat compartment, a gradual increase in PCB153 concentration is seen up to day 13. Fat compartment represents the biggest depot of PCB153 among all the tissues. Model structure assuming a diffusion-limited transport of PCB153 in fat tissue compartment gave a better description of the data. The permeability coefficients

obtained by fitting is much smaller than the blood flow rate (e.g., PAF/QF= 0.09), which is consistent with the model assumption that tissue uptake in the fat compartment is diffusion-limited. Parameters describing the oral absorption of PCB153 were obtained via model fitting to the fat concentration data (Table 3.1). Thus, as shown in Figure 3.2 and Figure 3.3, our modified PBPK model was able to describe time-dependent tissue concentration changes following two different exposure routes under two different doses.

(2) A PBPK model for describing PK of PCB153 in lactating mice and suckling pups. Taking the parameters estimated from non-pregnant model, with additional consideration on critical physiological changes during lactation (e.g., increased volume and blood flow in mammary tissues, milk secretion, and increased cardiac output), we derived a PBPK model for describing pharmacokinetic behavior of PCB153 in lactating mice and suckling pups. As shown in Figure 3.4, the lactational PBPK model was able to describe time-dependent concentration changes in fat, liver and brain tissues of dams, and liver and brain compartments of suckling pups. Similar to what is observed in non-lactating mice, the predicted peak concentration of PCB153 in liver and brain is achieved during the first 2 days after the dosing, followed by a gradual decline thereafter. Fat compartment also represents the biggest depot of PCB153 among all the tissues in lactating dams (Figure 3.4). The lactational PBPK model described an approximately 40 % of dam's PCB153 burden being transferred into suckling pups by PND 14.

(3) A PBPK model for describing lactational transfer of PCB153 with or without PCB126 in mice. To reflect the potential changes in lipoprotein lipase by

PCB126, we evaluated changes in diffusion permeation constants in the fat compartment (PAFC) of the model. As LPL is a membrane-bound protein, we assume that lipoprotein lipase changes would only impact the chemical diffusion process. Thus, we described chemical transfer in the fat tissue as diffusion-limited process. After incorporating changes of PAFC by PCB 126 into the model, we successfully described pharmacokinetic changes of PCB 153 in non-pregnant mice (Lee et al., 2002). In lactating mice and suckling pups, there are no statistically significant differences of PCB 153 levels in the liver and the fat between the groups with and without co-exposure of PCB 126. However, the mean concentration in the liver and the fat at each time point was different between these groups. Thus, we evaluated whether same decrease in PAFC and increase in PL, as seen in non-pregnant mice was able to improve the description on kinetic changes of PCB153 upon co-exposure of PCB126. In figure 3.5a and Figure 3.5b, we presented the simulation results, which suggested that PCB 126 might exert same effects in lactating mice and suckling pups as in non-pregnant mice.

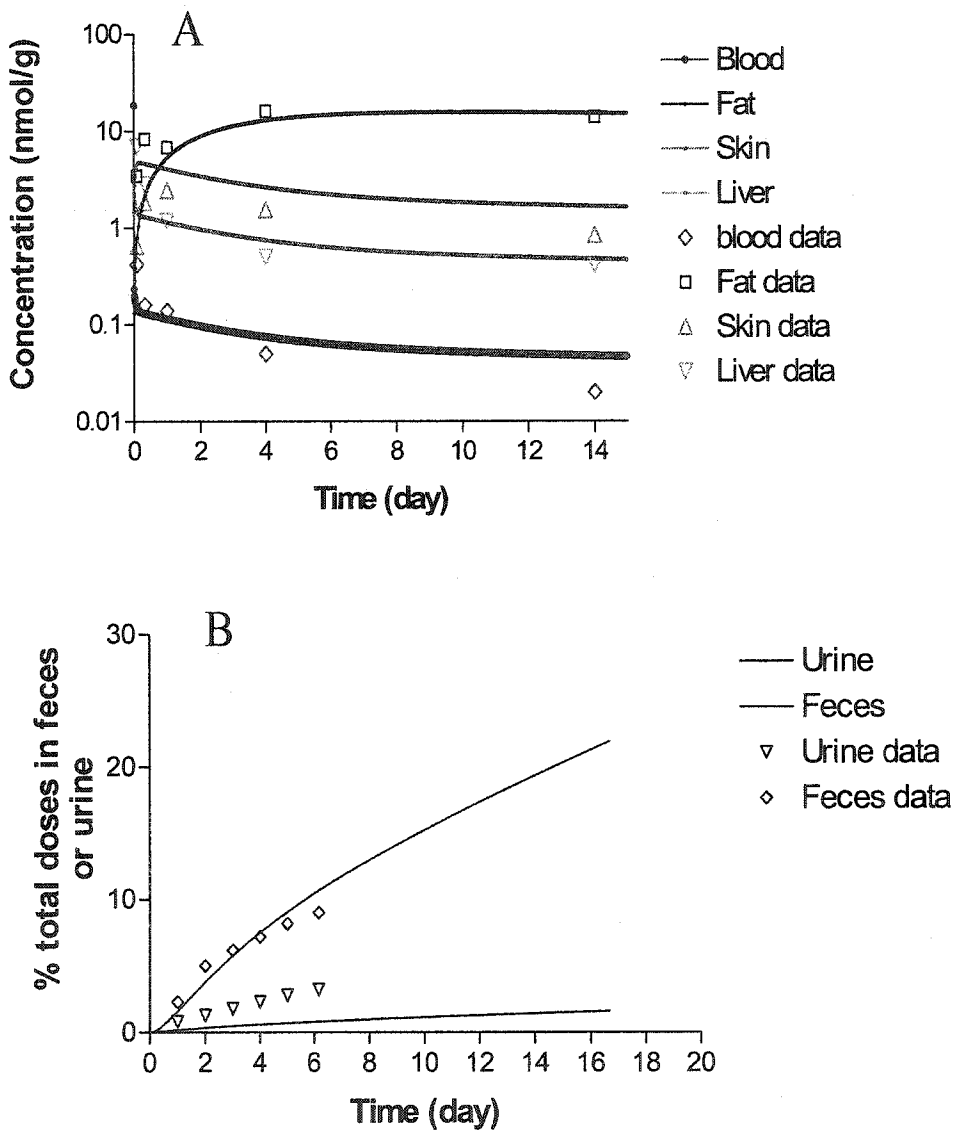

Fig 3.2. Simulation results following 0.6 mg/kg IV dose of PCB153. (a)Blood, fat, skin and liver tissue concentrations of PCB153 (b) fecal and urine excretion fraction.

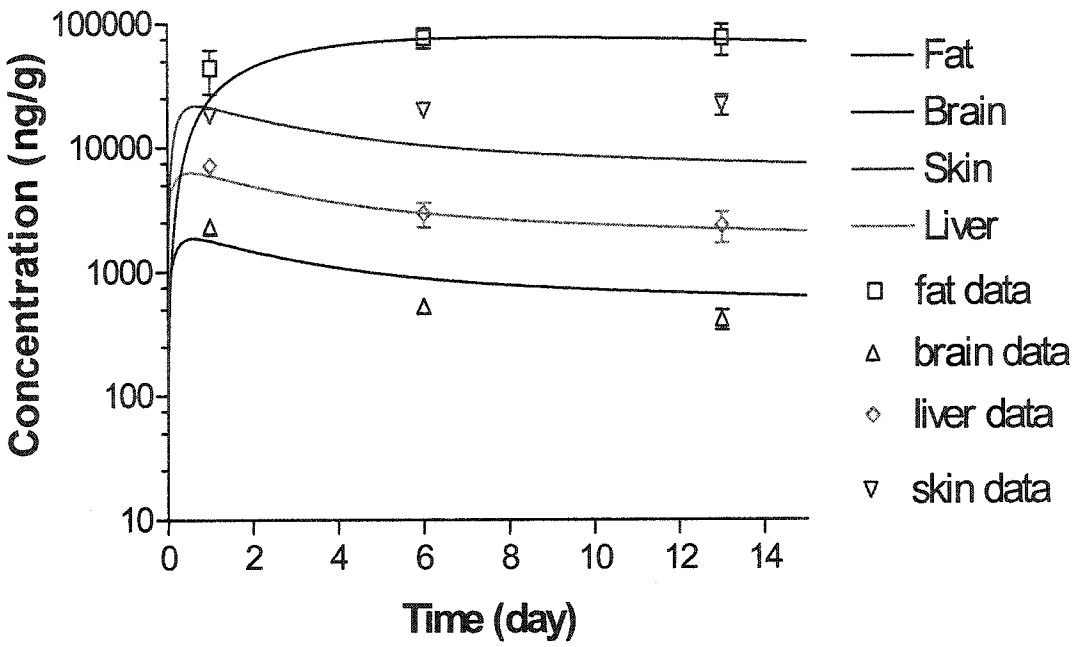

Fig. 3.3. Simulation results of PCB153 following 20 mg/kg PCB153. Female C57/BL6 non-lactating mice were dosed with a single oral gavage dose of 20 mg/kg PCB153. Solid circles shown are experimental measurements of blood, fat, skin and liver tissue concentrations of PCB153. Solid lines show simulation results from the PBPK model.

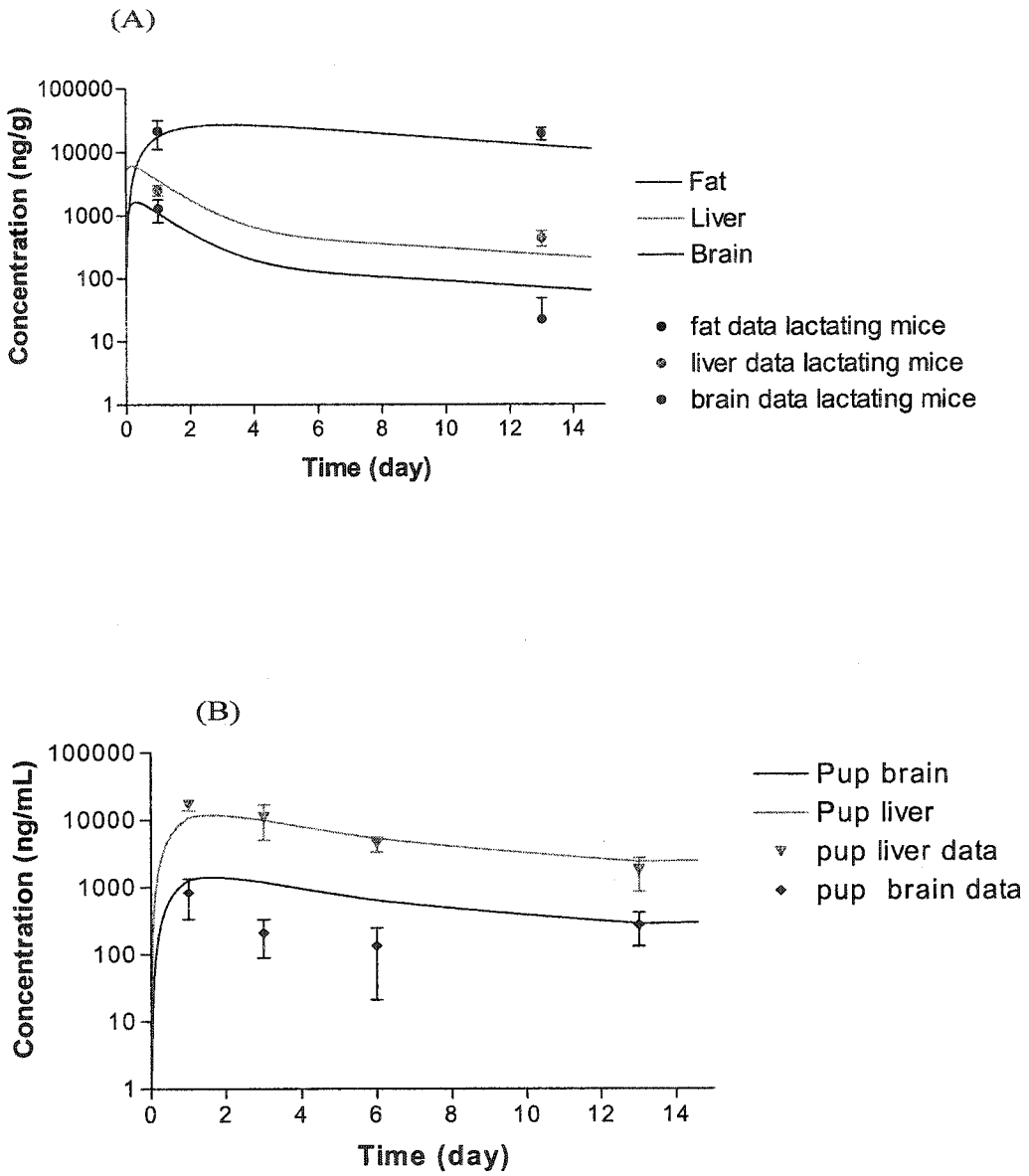

Fig. 3.4. Comparison of simulation results with experimental tissue concentration of PCB153 following an oral dose of 20 mg/kg PCB153 in lactating mice. Solid circles shown are experimental measurements of tissue concentrations of PCB153. Solid lines show simulation results from the lactional PBPK model. (a)Dams (b) pups.

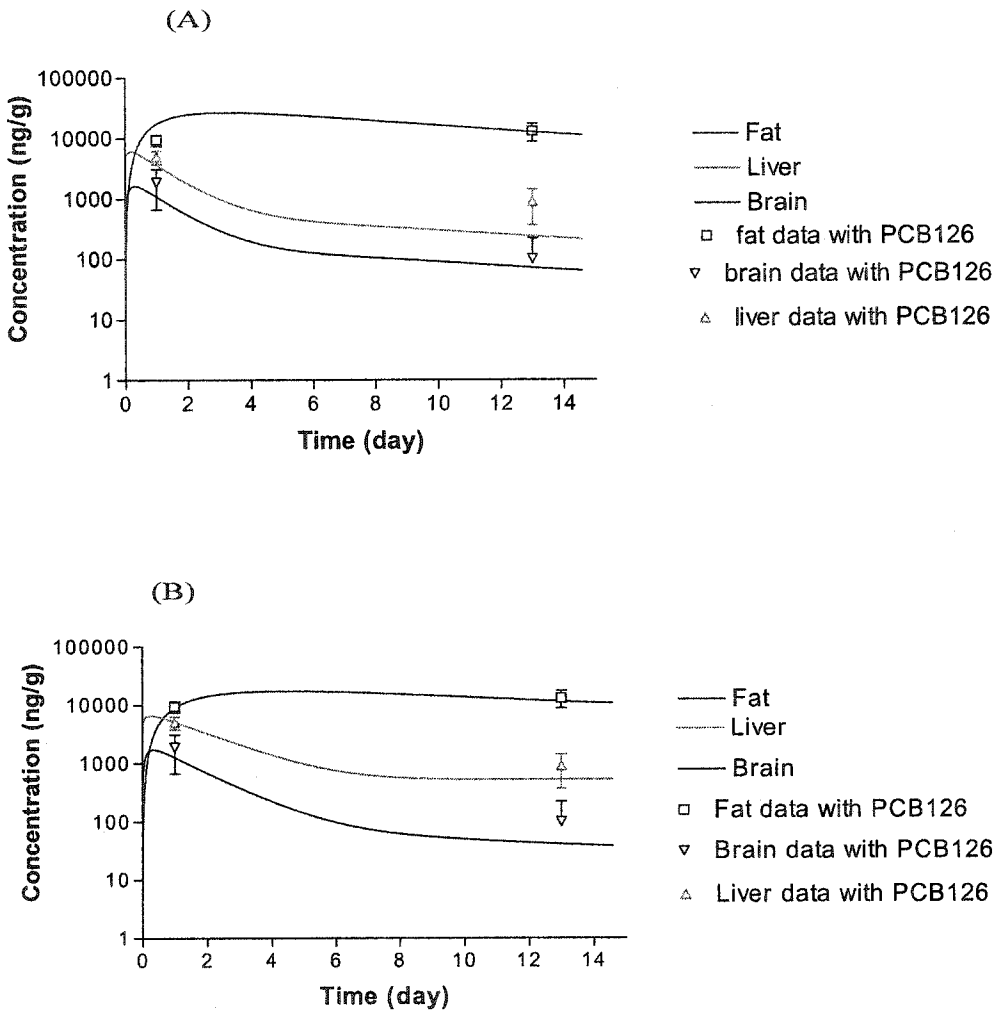

Fig. 3.5. PBPK simulation of PCB153 tissue concentration profile upon co-exposure of PCB126 in non-lactating and lactating mice. Female C57/BL6 mice were dosed with a single oral gavage dose of 20 mg/kg PCB153 and 0.2 mg/kg of PCB 126. Solid lines show simulation results from the PBPK model. (a) lactating mice, without incorporating effects of PCB126 in the lactational PBPK model (b) lactating mice, with incorporating effects of PCB126 in the lactational PBPK model.

DISCUSSION

We present here a PBPK model to describe the pharmacokinetics of PCB153 in lactating mice and suckling pups. Suckling offspring accumulated PCB153 through the milk from an exposed mother. This lactational PBPK model described the mass transfer of PCB153 into the developing organism during lactation by incorporating changes in the volume and blood flow into mammary tissues, and additional mechanistic changes in the movement of the PCB from adipose tissue to the mammary gland. We further used this model to understand the kinetic change of PCB153 with or without co-exposure of PCB126 during the lactational stage. In non-pregnant mice, a necessary parameter change in fat and liver compartment of the PBPK model was required to describe the observed time-course changes in PCB153 concentration upon co-exposure to PCB126. Similar to the observation in non-pregnant mice, incorporation of these parameter changes in a lactational PBPK model could also be used to better describe the mean tissue concentration changes in lactating mice upon co-exposure to PCB126.

A Lactational PBPK Model. One of the objectives of developing a lactational PBPK model for PCB153 is to study the lower tissue concentration burden in fat and liver in lactating mice compared to non-pregnant mice as seen in the present study and others (Vodicnik and Lech, 1980; Lee et al., 2002). It has been known that LPL undergoes reciprocal regulation in adipose tissue and mammary tissue during late pregnancy and lactation stage. There is an increase in mammary LPL activity throughout pregnancy, mirrored by a decline in the level in adipose tissue. These changes play an important role in the fate of circulating triglycerides, which are diverted from the uptake by

adipose tissue to the uptake by the mammary gland for milk synthesis. The capacity to synthesize fatty acids in mammary gland is maximally achieved during lactation. Because PCB153 is known to be bound with VLDL, a major substrate for LPL in mammary glands, an increased uptake of VLDL into mammary tissues would account for an increased uptake of PCB153 into mammary glands during late pregnant and lactational stage. We were able to incorporate these physiological changes and describe the kinetic changes in mammary gland/milk compartment and transfer dynamics of PCB153 from mammary tissue/milk compartment to suckling pups.

Modeling Effects of PCB 126 on PCB 153 Disposition: Implications of Ah-receptor Activation. Consistent with a previous study (Lee et al., 2002), the current modeling approach showed that changes in both PL and PAFC in the PBPK model were needed to describe PCB153 concentration profiles in non-pregnant mice upon co-exposure with PCB126. Incorporation of these parameter changes in a lactational PBPK model also significantly improved model description of the mean tissue concentration data in lactating dams upon co-exposure with PCB126. While the changes in lipid content in the liver is increasing over time, the rapid introduction of changes in PAFC is consistent with the experimental observation that reduction of adipose tissue LPL began as soon as 1 hr after TCDD administration, attained maximum depression after 2 days, and remained at that level throughout the 10-day observation period (Olsen et al., 1998). Inhibition of LPL activity with accompanying changes in hepatic contents has also been observed following exposure to TCDD-like PCB congeners as well other chemicals, including pesticides (Noren et al., 1999; Prabhakaren and Devi, 1993). For example, the incorporation of [1,2-

14C]acetate into the hepatic lipids was stimulated by both HCH and malathion, suggesting a higher rate of lipid synthesis in the liver. Future experiment to validate the prediction on the effect of PCB126 will include measurement of LPL activity in fat and mammary glands and lipid content in the liver.

Other researches on TCDD and co-planar PCB congeners suggested that most toxicities were caused by Ah-receptor activation (Mimura and Fujii-Kuriyama, 2003). Our studies also suggested that pharmacokinetic changes of PCB 153 co-exposed with PCB 126 may be caused by Ah-receptor activation. One review article pointed out that Ah-receptor activation induces many genes involving in metabolism (e.g., CYP 1A1/2, GST-Ya, UDPGT), cell proliferation (e.g., TGF-β, IL-1β), cell cycle (e.g., p27, jun-B), and apoptosis (e.g., Bax) (Mimura and Fujii-Kuriyama, 2003). In other studies, Ah-receptor activation may induce some kinases involved in growth factor singal transduction. These effects will be related to LPL, LDL receptors, glucose transporters, vitamin C uptake, and insulin secretion, ultimately affecting carbohydrate and lipid metabolism (Matsumura, 1995). Further studies may be needed to reveal how Ah-receptor signal transduction affects LPL activity.

Applications of Current model. A potential application of the current model would include risk management for pharmaceuticals that can be transferred to developing organism via breast-feeding. Plasma concentration in lactating mothers does not necessarily reflect the exposure in feeding babies. As increasing number of lipophilic drugs are to be used during late pregnancy and breast-feeding periods, estimation of drug concentrations in milk via a validated model would greatly facilitate drug labeling and risk management. For environmental chemicals, a tool

such as PBPK model can be potentially used to improve risk assessment of PCB mixture by predicting target tissue dosimetry of PCB congeners during critical developmental period. It is our hope that this PBPK model will encourage the use of pharmacokientic model in designing developmental studies for PCB153 or other PCB congeners.

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CHAPTER 4

Physiologically-based Pharmacokinetic Modeling of Pharmacokinetic Interactions for the Lactational Transfer of MeHg and PCB Congeners

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ABSTRACT

A PBPK model was developed to evaluate pharmacokinetic interactions for the lactational transfer of MeHg and PCB congeners (PCB 153, PCB 126) in mice. Some published studies have suggested: (1) MeHg and PCB congeners could affect the plasma levels of albumins and lipoproteins. (2) Most MeHg could bind to albumins in plasma, and about 60% of PCBs could bind to lipoproteins in plasma. In order to investigate the effects of lipoproteins and albumins on the lactational transfer of MeHg and/or PCBs and potential pharmacokinetic interactions, binding of PCB congeners to lipoproteins and binding of MeHg to albumins in plasma were incorporated into the model. Three hypotheses regarding the roles of lipoproteins and albumins were tested using PBPK modeling: First, chemicals could be transferred to the pups only bound with transport proteins. Second, unbound chemicals could be transferred to the pups. Third, the amount of lactational transfer of MeHg or PCBs may be changed only if co-exposure to these chemicals affects the levels of transport

proteins. Simulation results showed that the levels of lipoproteins and albumins were important factors determining the amounts of lactational transfer of MeHg and PCB congeners in all hypotheses. The experimental results using lactating mice and their pups showed that co-exposure with PCB congeners increased the lactational transfer of MeHg to the pups and compensated the plasma levels of albumin, which decreased by the exposure of MeHg only. These results were matched with the simulation results, suggesting that the first hypothesis may describe mechanism of the lactational transfer of MeHg and the third hypothesis may account for pharamacokinetic interactions between MeHg and PCB congeners. Further refinement of the models quantitatively described the pharmacokinetic changes for MeHg by co-exposure with PCBs in both maternal and pup's tissues. Our approach may improve risk assessment for the mixture of MeHg and PCBs in developing organisms.

INTRODUCTION

As described in previous chapters, both MeHg and PCBs are ubiquitous environmental contaminants and are recognized as representative neurotoxicants (Kodavanti *et al.*, 1998; Myers *et al.*, 2000). Developing brain is highly susceptible to both toxicants because of rapid growth and development (Abrha and Raghavan, 2000; Boersma and Lanting, 2000). Epidemiological studies have revealed that the babies and children born at MeHg and/or PCB contaminated sites showed reduced cognitive function and disrupted neurobehavioral characteristics even if their mothers and adults didn't show any indications of neurotoxicities (Buck, 1996; Mahaffey, 2000). Animal studies have suggested that perinatal exposure to MeHg and PCBs

may cause neurofunctional deficits including interruption in learning and memory (Newland and Paletz, 2000).

In addition, interactive effects between MeHg and PCBs have been demonstrated in some studies. In an epidemiologic study, neurobehavioral deficits in children indicated a possible interaction between MeHg and PCBs when they were exposed to the highest concentration of mercury and PCBs through perinatal exposure (De Guise *et al.*, 2001). Bemis and Seegal showed that co-exposure to MeHg and PCBs affects cytosolic calcium homeostasis in a non-additive manner and reduces dopamine content in brain synergistically (Bemis and Seegal, 1999; Bemis and Seegal, 2000).

Pharmacokinetic interactions between PCB congeners have been previously investigated in both lactating mice, their pups, and non-lactating mice (van Birgelen *et al.*, 1996; van der Plas *et al.*, 1988; Lee et al., 2002). However, no studies focusing on the pharmacokinetic interactions between MeHg and PCB congeners during lactation have been reported despite the potential importance of these interactions. The present study aimed to investigate pharmacokinetic interactions between MeHg and PCB congeners (i.e., PCB 153 and PCB 126) during the lactational period and to reveal the mechanisms of these interactions with the aid of PBPK modeling in addition to experimental approaches. This approach will provide useful information on risk assessment of environmental toxicants or drugs to which the developing organism is potentially exposed during critical period.

MATERIALS AND METHODS

Chemicals. PCB 153 and PCB 74 were purchased from Ultra Scientific (North Kingtown, RI). PCB 126 was obtained from Accustandard (New Haven, CT). The purities of all congeners used were over 98 %, which was confirmed by both vendors. MeHg was purchased from Sigma-Aldrich Chemical (St. Louis, MO). Pentane (HPLC grade), sea sand, and diethylether (> 99% purity) were purchased from VWR Scientific (Denver, CO). Sodium anhydrous sulfate and Florisil® (pesticide residue grade, 60-100 mesh) were purchased from Alltech Associates (Deerfield, IL).

Animal treatments. C57 BL/6 male and female mice were purchased from Harlan Sprague Dawley Laboratory (Indianapolis, IN) and housed at Painter Center in Colorado State University. The mice were maintained on a 12-hr light/dark cycle at a constant temperature of 25 °C and humidity of 55%. Mating took place at Painter Center in Colorado State University according to the following breeding protocol. Briefly, two female mice were placed together with one male in a cage for 4 consecutive days. We assigned the day after mating as GD 0. The pregnancy rate was about 60%. The animals were weighed every three days during gestation. On PND 0, 7, and 14, the offspring were counted and inspected for signs of overt toxicity.

On PND 1, lactating mice were exposed to MeHg alone (1 mg/kg bw), a mixture of PCB 153 (20 mg/kg bw) and PCB 126 (0.2 mg/kg bw), or a mixture of MeHg, PCB 153, and PCB 126 through oral gavage. Corn oil was used as vehicle to dissolve the chemicals. The dosing time was between 8:00 and 9:00 a.m. The concentration of each dosing solution was confirmed by atomic absorption spectrometry and gas chromatography. At 1, 3, 6, and 13 days after oral gavage,

lactating mice and their pups were anesthetized using isofluorane and then euthanized. The tissues of lactating mice (liver, fat, kidney, blood, and brain) and pups (liver, brain, kidney, and the remaining carcass) were collected from each animal. Non-pregnant mice were treated the same way as lactating mice and their tissues were collected at 1, 6, and 13 days after treatment. All samples were frozen with liquid nitrogen and stored at –70 °C until analysis.

Chemical analyses. Analyses of PCB congeners were based on the method by Lee et al. (2002). In brief, tissue samples were digested by 60% of sulfuric acid and extracted by pentane. After cleaning up extracts with Florisil, concentrations of PCB 153 or PCB 126 in samples were determined using a HP-5890 Series II Plus gas chromatography with a ECD detector (Hewlett Packard, Wilmington, DE). The analyses of mercury were based on the method by Environmental Protection Agency (EPA) (Smoley, 1992). Briefly, tissues samples were digested by the mixture of nitric acid and sulfuric acid and completely oxidized by potassium permanganate and potassium persulfate. The concentration of mercury was determined using a cold vapor atomic absorption spectrometry (Varian, Sugarland, TX) according to the method provided by vendor.

Measurement of albumin and lipoproteins in blood. Albumin measurement was based on the BCG method reported previously (Tietz, 1970). Briefly, serum was collected by centrifugation of maternal blood. A 18 *u*l of each serum was mixed with 2.1 ml of BCG reagent provided by Amresco (Solon, OH). The absorbance was measured at 600 nm according to the method provided by the vendor. Lipoprotein measurement was based on the separation by electrophoresis previously reported

(Converse and Skinner, 1992). Briefly, serum samples were applied to the lipoprotein gel provided by Helena laboratories (Beaumont, TX). After separation, gels were stained using fat Red 7B (0.1% in 95% methanol). The density of each gel band was quantitated by Alpha-Imager 2200 (Alpha Innotech Corp., CA).

PBPK model development. Previously, PBPK models describing lactational transfer of MeHg or PCB congeners were published (Byczkowski and Lipscomb, 2001; Ou *et al.*, 2001). The main purpose of our modeling is to provide a mechanistic explanation for pharmacokinetic interactions between MeHg and PCB congeners during lactational period. Thus, the modeling approach began with the comprehensive understanding of published models. After that, all parameters were reevaluated against the experimental data. To incorporate mechanistic information, we added additional compartments involving serum albumin and lipoprotein pools. Finally, an integrated model was developed to combine all information in order to describe pharmacokinetics of both MeHg and PCB congeners. In the following sections, we described the modeling process in detail.

Model structure. The basic model structure for MeHg and PCBs was obtained from previous publications (Byczkowski and Lipscomb, 2001; Ou *et al.*, 2001). We combined these model structures in order to describe the disposition of both MeHg and PCB congeners (Figure 4.1.). The percentage of uriny and fecal excretion was verified with literature data. Mass transport was first described as depicted in the original articles. Later, we evaluated chemical uptake as either a flow-limited process or a diffusion-limited process and adopted one simulating chemical disposition more accurately. The blood compartment was separated into a serum compartment and a

transport protein compartment (i.e., albumin and lipoprotein pools). Chemical transfer from the mother to the pups was based on two hypotheses: First, chemicals bound with transport proteins could be transferred from mother to the pups. In this scenario, chemicals in transport protein compartment could be moved to the mammary gland, and subsequently transferred to the pups. Second, unbound chemicals could be transferred from mother to the pups. In this scenario, chemicals in the serum compartment could be moved to the mammary gland, and subsequently transferred to the pups. In the following section, we described mathematical expressions for these compartments including chemical transfer.

Mathematical expression of the model. The mathematical expression of the model was obtained from a mass balance on each compartment. Tissue mass balance equations were formulated as outlined in Ramsey and Andersen (Ramsey and Andersen, 1984). Equation (1) represents a tissue mass balance equations for a flow-limited transport process.

$$dAi/dt = Q_i^* (C_B - C_{Vi}) \quad (1)$$

Where Q_i is blood flow to ith tissue; Ai is amount in ith tissue. C_B is aterial blood concentration; Cv_i is venous blood concentration leaving ith tissue. C_{Vi} = Ci /Pi, where Pi is tissue i/blood partition coefficient, Ci is concentration in ith tissue. C_i = A_i / V_i; where V_i is the volume of ith tissue (litters). Equation (2)-(3) are the tissue mass balance equations for a diffusion-limited transport process.

$$dA_{iB}/dt = Q_i^* (C_B - C_{Vi}) + P_{Ai}^* ((C_i/P_i) - C_{Vi}) \quad (2)$$

$$dA_i/dt = P_{Ai}^* (C_{Vi} - (C_i/P_i)) \quad (3)$$

Where Qi is blood flow to ith tissue; Vi is the volume of ith tissue; A_{iB} is amount in the blood compartment of ith tissue.

The equation for the mammary tissue/milk is described using two different scenarios, depending on the hypotheses of chemical transfers. In equation (4) and (5), we describe the process in which chemicals are transferred to the pups without the binding of transport proteins.

$$dA_{MA}/dt = Q_{MA}* (C_B - C_{VMA}) - dAsuck/dt \quad (4)$$

Where C_{VMA} is venous blood concentration leaving mammary/milk tissue. The second term (dAsuck/dt) is identical to the pup litter suckling rate.

$$dAsuck/dt = C_{MA}*K_{MILK} \quad (5)$$

K_{MILK} is the milk transfer rate and was assumed to equal the milk production rate.

In equation (6) and (7), we described that chemicals are transferred to the pups with the binding of transport proteins.

$$dA_{MA}/dt = Q_{MA}* (C_{Bound} - C_{VMA}) - dAsuck/dt \quad (6)$$

Where C_{Bound} is chemical concentration bound with transport proteins in blood.

$$dAsuck/dt = C_{MA}*K_{MC} \quad (7)$$

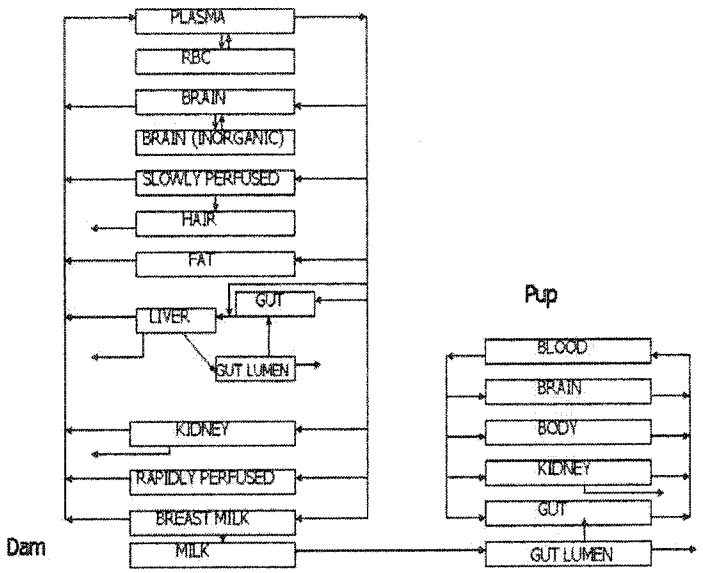
K_{MC} is the chemical transfer rate from milk and was assumed to be dependent on the amount of transport proteins in blood and milk production rate.

For the description of PCB disposition, the partition coefficient in the liver (PL) was modified to a time-dependent equation,

$$PL = 10 + KFL*T \quad (8)$$

where KFL is the coefficient for time-dependent increase of partition coefficient and T is the time (minutes) after PCB 153 exposure, where 0<=T<=504 hr (14 days).

(A)



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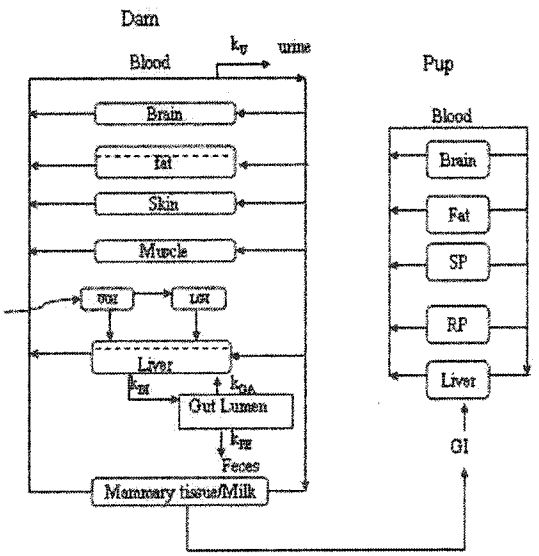


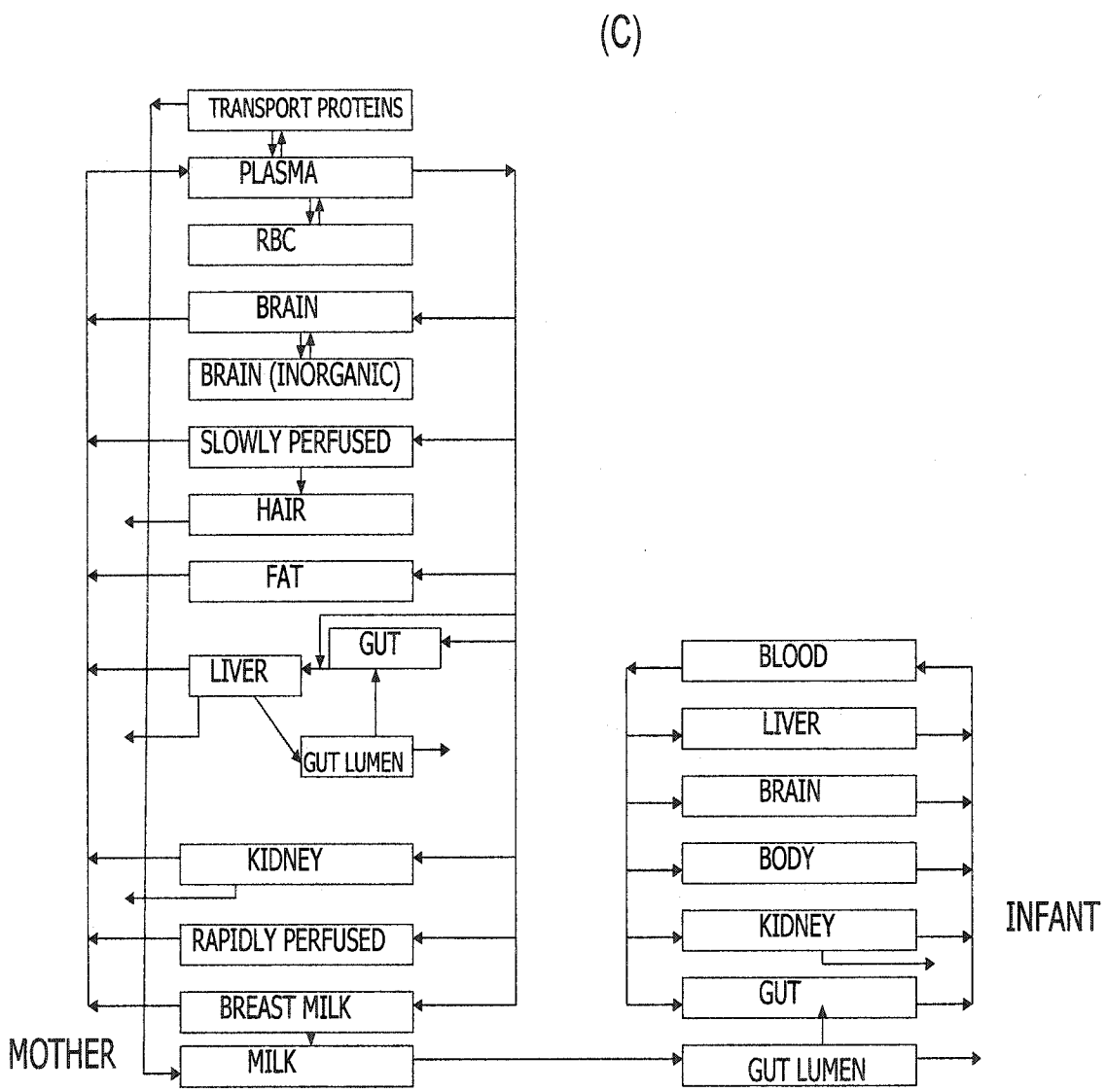


Fig. 4.1. Schematic diagrams of the PBPK models describing the lactational transfer of MeHg and/or PCB congeners. (A) MeHg (Byczkowski and Lipscomb, 2001). (B) PCB congeners (Ou *et al.*, 2001). (C) Final model structure describing lactational transfer of both MeHg and PCB congeners.

The equation describing body weight growth of the lactating pup (BWP) was adapted from previous literature (Corley *et al.*, 2003):

$$BWP = 0.005 + ADULT*[(\exp(AGE) - 1)/(\exp(HALF) + (\exp(AGE) - 1))] \quad (9)$$

where HALF is the one month after birth in mice, ADULT is the normal weight of adult mouse, and AGE is the month after birth in mice.

Parameter values. Physiological constants such as blood flow, tissue volume, and partition coefficients were taken directly from the published literature (Brown *et al.*, 1997). Metabolism and elimination constants describing fecal and urine excretion were estimated based on fitting of the model to the data previously published (Byczkowski and Lipscomb, 2001; Ou *et al.*, 2001). All other parameters showing changes during lactational period followed the values or mathematical expressions in previous publications (Byczkowski and Lipscomb, 2001; Ou *et al.*, 2001). All parameters mentioned above were presented in Table 4.1.

Simulation software. All PBPK model construction, simulation, and parameter estimations were performed using the Berkeley Madonna software package (version 8.01 for Windows, Kagi Shareware, Berkeley, CA). Sensitivity analysis was performed to determine sensitivity of model output to the parameters according to the method reported previously (Reddy *et al.*, 2003).

Statistical analysis. Differences of tissue concentration between samples from various treatment groups and time points were tested for significance by two-way ANOVA, followed by Fisher's multiple comparison test. All analyses were performed with the statistical software, Minitab ($P < 0.01$; Windows version 12.0).

Table 4.1. PBPK model parameters

Parameter	Abbreviation	Value	Parameter estimation
	Physiological Parameters		
	Maternal Parameters		
Maternal Body weight (kg)	BW	0.025	Literature[a]
Fraction of brain	BF	0.020	Literature[a]
Fraction of plasma	BLF	0.022	Literature[a]
Fraction of RBC	RBCF	0.027	Literature[a]
Fraction of fat	FF	0.056	Literature[a]
Fraction of hair	HF	0.002	Literature[a]
Fraction of rapidly perfused	RF	0.100	Literature[a]
Fraction of slowly perfused	SSF	See legend[b]	Literature[a]
Fraction of gut	GF	0.021	Literature[a]
Fraction of gut lumen	IF	0.021	Literature[a]
Fraction of liver	LF	0.080	Literature[a]
Fraction of kidney	KF	0.0017	Literature[a]
Fraction of mammary gland	MAF	0.044	Literature[a]
Cardiac output (l/hr)	QC	16.5*BW**0.75	Literature[a]
Blood flow of brain	QBF	0.033	Literature[a]
Blood flow of fat	QFF	0.07	Literature[a]
Blood flow of kidney	QKF	0.091	Literature[a]
Blood flow of GI tract	QGF	0.141	Literature[a]
Blood flow of liver	QLF	0.159	Literature[a]
Blood flow of mammary gland	QMAF	0.1*QRF	Literature[a]
Blood flow of rapidly perfused	QRF	0.183	Literature[a]
Blood flow of slowly perfused	QSSF	See legend[c]	Literature[a]
	Pup's parameters		
Pup's Body weight (kg)	BWP	See Text	Literature[a]
Fraction of blood	BLPF	0.049	Literature[a]
Fraction of brain	BPF	0.020	Literature[a]
Fraction of kidney	KPF	0.0017	Literature[a]
Fraction of liver	LPF	0.080	Literature[a]
Fraction of gut	GPF	0.021	Literature[a]
Fraction of gut lumen	IPF	0.021	Literature[a]
Fraction of body	CARF	See legend[d]	Literature[a]
Cardiac output (l/hr)	QCP	18*BWP**0.75	Literature[a]
Blood flow of brain	QBPF	0.033	Literature[a]
Blood flow of kidney	QKPF	0.091	Literature[a]
Blood flow of liver	QLPF	0.159	Literature[a]
Blood flow of GI tract	QGPF	0.141	Literature[a]
Blood flow of body	QCARF	See legend[e]	Literature[a]
	Partition Coefficients of MeHg		
Brain	PB	3.0	Literature[f]
Slowly perfused	PS	2.0	Literature[f]

Fat	PF	0.15	Literature[f]
Liver	PL	3.0	Literature[f]
Gut	PG	1.0	Literature[f]
Kidney	PK	5.0	Literature[f]
Rapidly perfused	PR	1.0	Literature[f]
Mammary gland	PM	0.2	Literature[f]
	Partition Coefficients of PCB 153		
Brain	PB	2.5	Literature[g]
Fat	PF	300	Literature[g]
Skin	PS	35	Literature[g]
Muscle	PM	5	Literature[g]
Liver	PL	See Text	Literature[g]
Mammary gland	PM	80	Literature[g]
Rapidly perfused	PR	2.5	Literature[g]

[a]Brown *et al.*, 1997
[b]1 – (BF+BLF+RBCF+FF+HF+RF+GF+IF+LF+KF+MAF)
[c]1 – (QBF+QKF+QFF+QGF+QLF+QMAF+QRF)
[d]1 – (BLPF+BPF+KPF+LPF+GPF+IPF)
[e]1 – (QBPF+QKPF+QLPF+QGPF)
[f]Byckzowski and Lipscomb, 2001
[g]Ou *et al.*, 2001

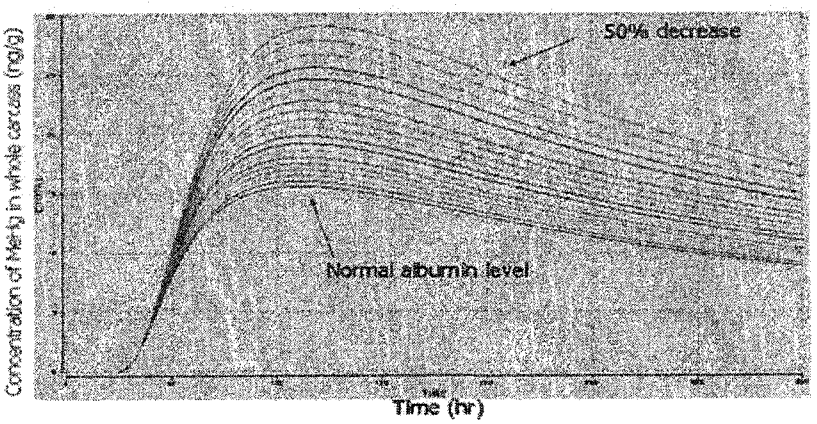
RESULTS

Hypothesis generation with PBPK modeling. Extensive literature searches revealed: (1) MeHg and PCB congeners could affect the plasma levels of albumins and lipoproteins (Matthews *et al.*, 1977; Yasutake *et al.*, 1989), (2) most MeHg could bind to albumins in plasma, and about 60% of PCBs could bind to lipoproteins in plasma (Vodicnik and Lech, 1980; Sundberg *et al.*, 1998), and (3) plasma albumin and lipoproteins could play a role in the lactational transfer of MeHg and PCB congeners (Spindler-Vomachka *et al.*, 1984; Sundberg *et al.*, 1998). We wanted to evaluate whether the effects of MeHg and/or PCB congeners on albumin and lipoproteins in maternal blood could affect the lactational transfer of these chemicals. To perform this evaluation, we constructed a PBPK model describing the lactational transfer of MeHg and PCB congeners based on the previous models (Byczkowski and Lipscomb, 2001; Ou *et al.*, 2001). We combined two models in order to describe the disposition of both MeHg and PCB congeners (Fig. 4.1.). To evaluate the role of albumin and lipoproteins on the lactational transfer of MeHg and PCB congeners, we incorporated the transport protein pool into the blood compartment. The physiological parameters in this compartment were adopted from those of albumins for the simulation of MeHg and lipoproteins for the simulation of PCB congeners based on the previous publications (Spindler-Vomachka *et al.*, 1984; McMullin *et al.*, 2003). We formulated two different hypotheses for the lactational transfer of MeHg and PCB congeners. The first hypothesis is that only unbound MeHg or PCB congeners will be transferred from the mother to the pups. This hypothesis was based on the literatures dealing with the lactational transfer of drugs and environmetal

contaminants (Corley *et al.*, 2003). For the modeling of this scenario, we represented that the chemicals in serum will be moved into the mammary gland, where the chemicals will be transferred to the milk, and subsequently transferred to the pups. The second hypothesis is that MeHg bound with albumins and PCB congeners bound with lipoproteins will be transferred from the mother to the pups. This hypothesis was based on the previous publications focusing on the lactational transfer of MeHg or PCB congeners (Vodicnik and Lech, 1980; Sundberg *et al.*, 1998).

PBPK simulation results for the lactational transfer of MeHg and PCB congeners were presented in Fig. 4.2. In Fig. 4.2. (A) and (B), we showed the simulation results for the lactational transfer of MeHg against the level of albumins in maternal blood. The result of evaluating the first hypothesis suggested that the lactational transfer of MeHg could be inversely proportional to the levels of albumin in maternal blood. The result of evaluating the second hypothesis suggested that the lactational transfer of MeHg could be directly proportional to the levels of albumin in maternal blood. Both results showed that the lactational transfer of MeHg may be dependent upon the levels of albumin in maternal blood. In Fig. 4.2. (C) and (D), we showed the simulation results for the lactational transfer of PCB 153 against the level of lipoproteins in maternal blood. Overall, the simulation results showed similar tendency to those of MeHg. In other words, the first hypothesis suggested that the lactational transfer of PCB congeners could be inversely proportional to the levels of lipoproteins in maternal blood. The second hypothesis suggested that the lactational transfer of PCB congeners could be directly proportional to the levels of lipoproteins in maternal blood.

(A)



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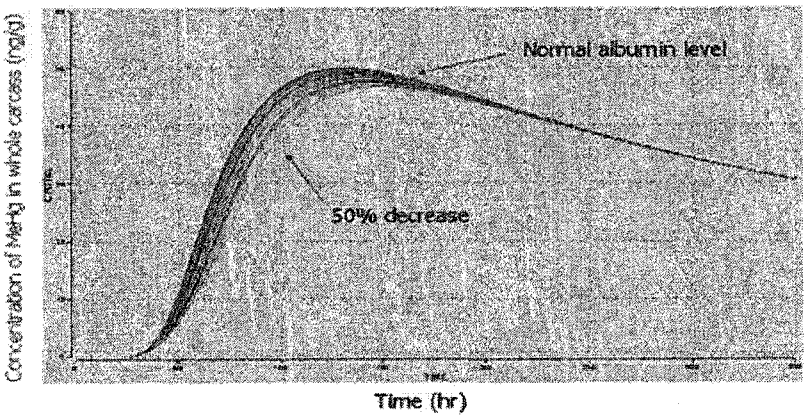

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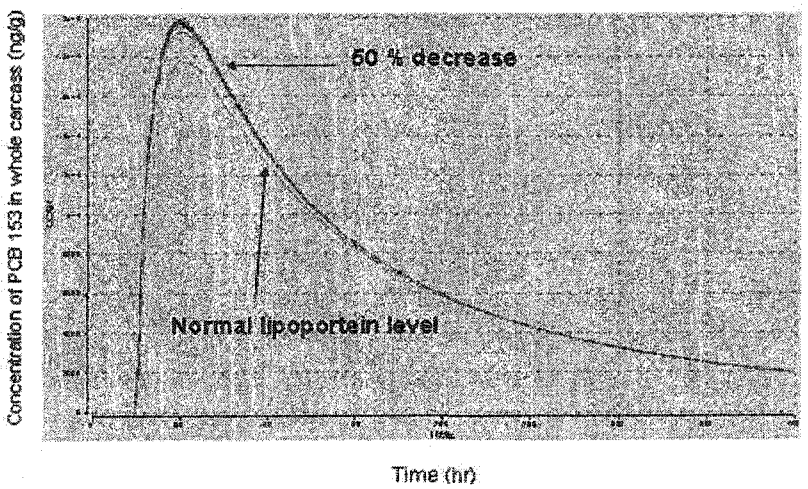

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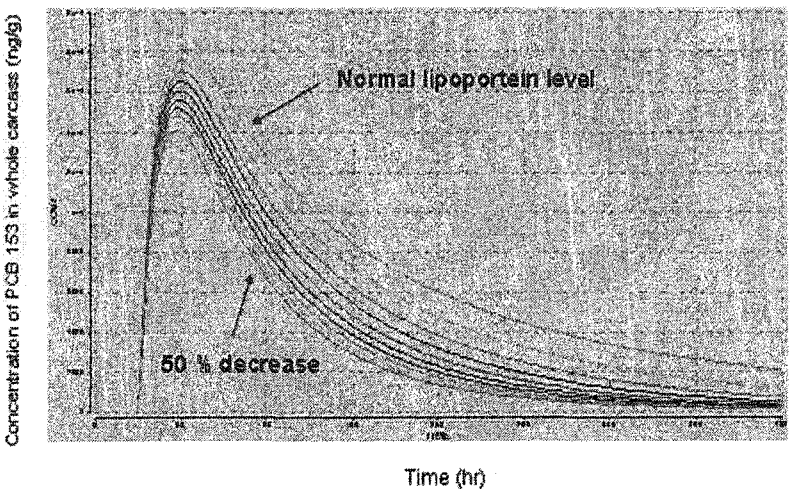


Fig. 4. 2. PBPK simulation results for the lactational transfer of MeHg and PCB congeners against the levels of transport proteins in maternal blood. (A) The simulation results for the lactational transfer of MeHg to the pups in the first hypothesis. Albumin levels varied from 50% to 100% of normal levels in mice. (B) The simulation results for the lactational transfer of MeHg to the pups in Second hypothesis. Albumin levels varied from 50% to 100% of normal levels in mice. (C) The simulation results for the lactational transfer of PCB 153 to the pups in first hypothesis. Lipoprotein levels varied from 50% to 100% of normal levels in mice. (D) The simulation results for the lactational transfer of PCB 153 to the pups in the Second hypothesis. Lipoprotein levels varied from 50% to 100% of normal levels in mice.

Through PBPK simulations, we could generate the following conjectures regarding pharmacokinetic interactions between MeHg and PCB congeners on the lactational transfer. First, co-exposure of MeHg and PCB congeners could affect the lactational transfer of one another in the case whereby they could affect the levels of transport proteins (i.e., albumin and lipoproteins) in maternal blood. Second, if PCB congeners could increase the levels of albumins in maternal blood, the lactational transfer of MeHg would decrease according to the first hypothesis and increase according to the second hypothesis. Third, the lactational transfer of PCB congeners will be affected by co-exposure with MeHg.

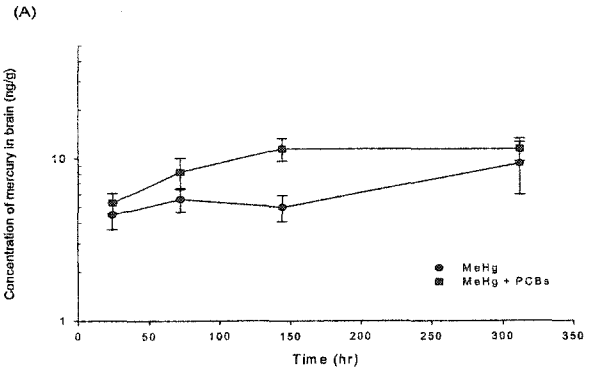
Experimental findings

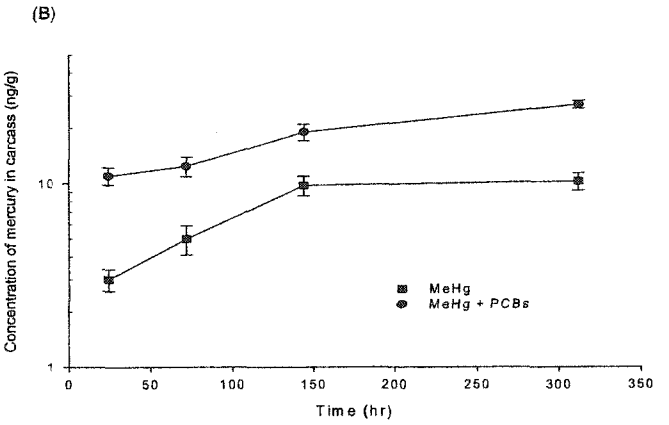
Tissue dosimetry of MeHg and albumin amounts. Fig. 4.3. showed the results of tissue levels of MeHg in the pups and the levels of albumin in the maternal blood. In Fig. 4.3. (A), we presented time-course concentration of MeHg in the pup's brain. The group exposed to MeHg only showed that the tissue concentration increased gradually from PND 2 through PND 14. The group exposed to MeHg + PCB congeners showed that the tissue concentration attained the maximal level at PND 7. At PND 7, the tissue concentration was higher in the group exposed to MeHg + PCB congeners than the group exposed to MeHg only at a statistically significant level ($p < 0.05$). Even though there were no statistically significant differences at other time points, the tissue concentration was higher in the group exposed to MeHg + PCB congeners. In Fig. 4.3. (B), we presented time-course concentration of MeHg in the pup's carcass. Like the case for the brain, the tissue concentration was higher in the group exposed to MeHg + PCB congeners than the group exposed to MeHg only.

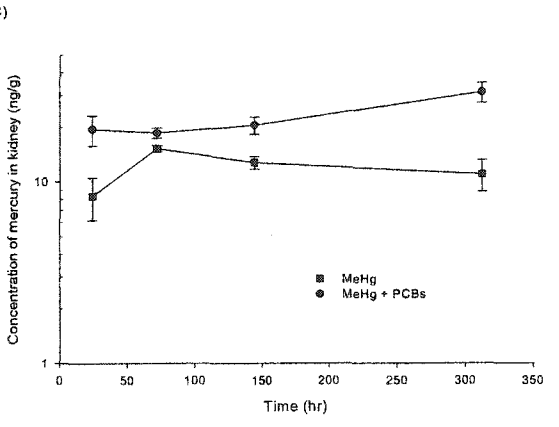
Moreover, the tissue concentrations were statistically different at all time points ($p < 0.05$). In Fig. 4.3. (C), we presented time-course concentration of MeHg in the pup's kidney. First of all, the mean concentration of MeHg in the kidney was higher than in other tissues, which suggested high affinity of mercury to kidney previously reported (Nielsen, 1992). Overall, the tissue concentration of MeHg was higher in the group exposed to MeHg + PCB congeners than in the group exposed to MeHg only. We noted that there were statistically significant differences between the groups at PND 7 and PND 14 ($p < 0.05$). The unique characteristic of tissue disposition in the group exposed to MeHg + PCB congeners was that tissue concentration increased until PND 14. This result suggested that co-exposure with PCB congeners elicited the longer retention of MeHg in pup's kidney. The above results demonstrated that the tissue concentrations of MeHg were higher in the group exposed to MeHg + PCB congeners than in the group exposed to MeHg only. In addition, these results suggested that co-exposure with PCB congeners to the lactating mice increased the lactational transfer of MeHg to the pups.

To investigate whether the increase of lactational transfer was associated with albumin levels in maternal blood, we compared the albumin levels in maternal blood between the group exposed to MeHg only and the group exposed to MeHg + PCB congeners. In Fig. 4.3. (D), we presented time-course concentration of albumin levels in maternal blood. There were statistically significant differences of albumin levels at PND 2 and PND 4 between the group exposed to MeHg only and the group exposed to MeHg + PCB congeners. At other time points, there were no statistically significant differences. At PND 2 and PND 4, the albumin levels were higher in the

group exposed to MeHg + PCB congeners than in the group exposed to MeHg only. These results supported that the second hypothesis could explain the pharmacokinetic interactions for the lactational transfer between MeHg and PCB congeners.







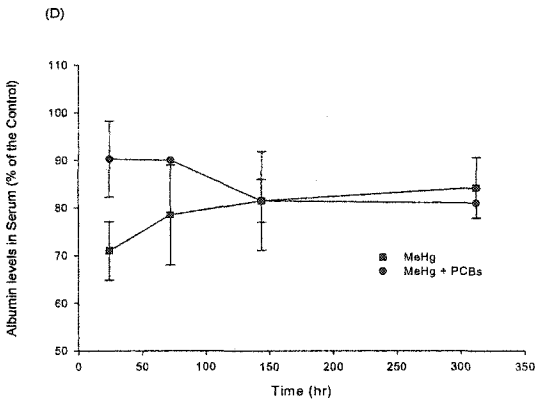


Fig. 4.3. The tissue concentrations of MeHg in the pup and the levels of albumin in maternal blood. (A) The time-course concentration of MeHg in pup's brain. (B) The time-course concentration of MeHg in pup's carcass. (C) The time-course concentration of MeHg in pup's kidney. (D) The time-course concentration of albumin in maternal blood.

In Table 4.2, we presented the time-course concentrations of MeHg in maternal tissues. Overall, the tissue concentrations of MeHg were higher in the group exposed to MeHg + PCB congeners than the group exposed to MeHg only. These results suggested that co-exposure with PCB congeners could increase the concentrations of MeHg in both maternal and pup's tissues.

Table 4.2. Summary of MeHg concentrations in the maternal tissues (ng/g tissue).*

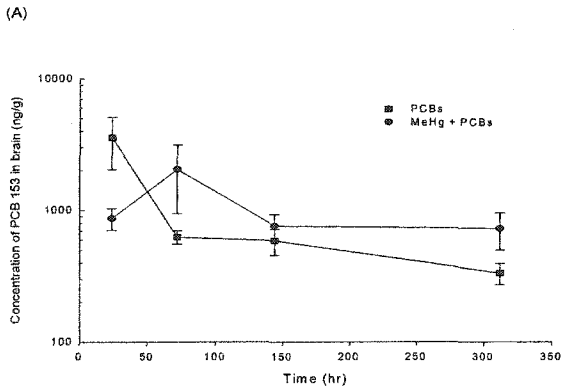
	Time	Lactating dams (MeHg only)	Lactating dams (MeHg + PCBs)
Kidney	Day 1	882±389	1354±700
	Day 3	1292±344	2096±516

	Day 6	711±90	1271±595
	Day 13	205±54	955±189
Blood	Day 1	177±3	418±35
	Day 3	143±16	358±160
	Day 6	115±40	376±180
	Day 13	39±2	117±30
Brain	Day 1	102±40	199±18
	Day 3	217±100	253±74
	Day 6	149±13	327±239
	Day 13	89±7	217±10

*All values reported here are mean±SD (n = 4)

Tissue dosimetry of PCB congeners and lipoprotein amounts. Fig. 4.4 showed the results of PCB congeners in the pups. In Fig. 4.4. (A), we presented time-course concentration of PCB 153 in pup's brain. The tissue disposition of the group exposed to PCB congeners only showed that the concentration of PCB 153 attained the maximum level at PND 2 and decreased after that. The tissue disposition of the group exposed to MeHg + PCB congeners showed that the concentration of PCB 153 attained the maximum level at PND 4 and decreased after that. The tissue levels at PND 4 showed statistically significant differences between the groups ($p < 0.05$). The tissue levels at other time points showed no differences between the groups. Time-course concentration of PCB 153 in the pup's carcass were shown in Fig. 4.4(B). The time-course changes of PCB 153 showed similar tendencies mentioned above. The tissue levels did not show any statistical differences between the groups.

In Fig. 4.4. (C), we presented time-course concentration of PCB 153 in the pup's liver. In both groups, the time-course changes of PCB 153 showed gradual decreases of tissue levels with time. The tissue levels showed statistically significant differences at PND 4 ($p < 0.05$). At other time points, there were no differences. In Fig. 4.4. (D), we presented time-course concentration of PCB 126 in pup's liver. Tissue levels gradually increased with time and attained maximal levels at PND 14 in both groups. Tissue levels were not statistically significant between the groups. In other tissues, we could not detect PCB 126 at all time points, which were consistent with our previous studies (Lee *et al.*, 2002). In summary, the lactational transfer of PCB congeners from mother to the pups was not different between the group exposed to PCB congeners only and the group exposed to MeHg + PCB congeners. However, the time to attain maximal concentration was different between the groups. We measured the lipoprotein levels in maternal blood. The levels were not different between the group exposed to PCB congeners only and the group exposed to MeHg + PCB congeners (Data not shown). These results were consistent with above results showing that the lactational transfer was not different between the groups.



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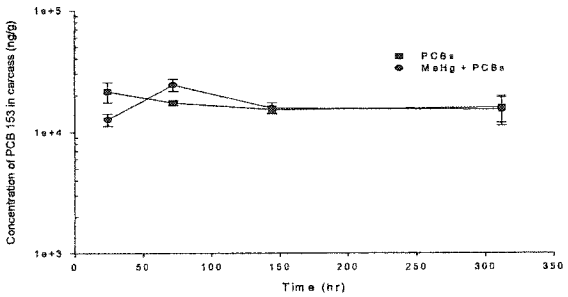

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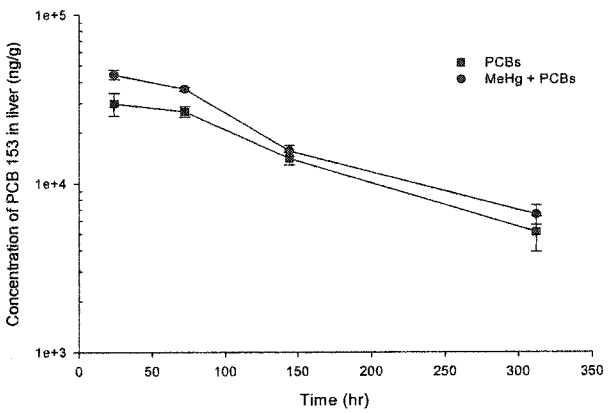

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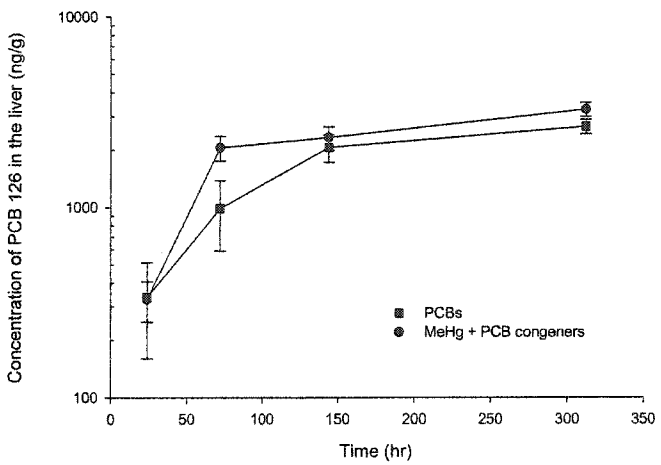

Fig. 4.4. The tissue concentrations of PCB congeners in the pup. (A) The time-course concentration of PCB 153 in pup's brain. (B) The time-course concentration of PCB 153 in pup's carcass. (C) The time-course concentration of PCB 153 in pup's liver. (D) The time-course concentration of PCB 126 in pup's liver.

In Table 4.3, we presented time-course concentrations of PCB congeners in maternal tissues. Overall, tissue concentrations of PCB congeners were not different between the groups at all time points.

Table 4.3. Summary of PCB 153 levels in maternal tissues (ng/g tissue).*

	Time	Lactating dams (MeHg only)	Lactating dams (MeHg + PCB congeners)
Liver	Day 1	5065	4256
	Day 3	3425±648	4461±453
	Day 6	1375±255	2426±155
	Day 13	1525±617	1279±898
Fat	Day 1	43030	35468
	Day 3	38908±4340	45727±2322
	Day 6	45110±2355	37913±1455
	Day 13	20688±5858	23296±6386
	Day 1	ND	ND

Brain	Day 1	ND	ND
	Day 3	819±25	645±38
	Day 6	ND	ND
	Day 13	389±197	124±58

*All values reported here are mean±SD (n = 4)

PBPK modeling: refinement and validation. In Fig. 4.5, we presented the refined simulation results of MeHg disposition in pup's tissues. For the refinement of the model described earlier, we optimized parameters reflecting the physiology and metabolism of growing mice. In addition, we decreased the formation constant of albumin in maternal blood according to the experimental findings. The model adequately described time-course tissue distributions of MeHg in the pups. Compensation of albumin formation to the normal level was able to describe pharmacokinetic changes induced by co-exposure with PCB congeners. These results were consistent with experimental findings and our hypothesis.

In Fig. 4.6, we presented simulation results of PCB 153 in pup's tissues. As mentioned above, lactational transfer of PCBs was not affected by co-exposure with MeHg. Flow-limited transfer failed to describe the time delay of PCB 153 by co-exposure with MeHg. Diverse modeling approaches were attempted and diffusion-limited uptake in the pup's tissues adequately simulated the time delay caused by co-exposure with MeHg. These results suggested that co-exposure with MeHg affected the uptake mechanism of PCB 153 in the pup's tissues.

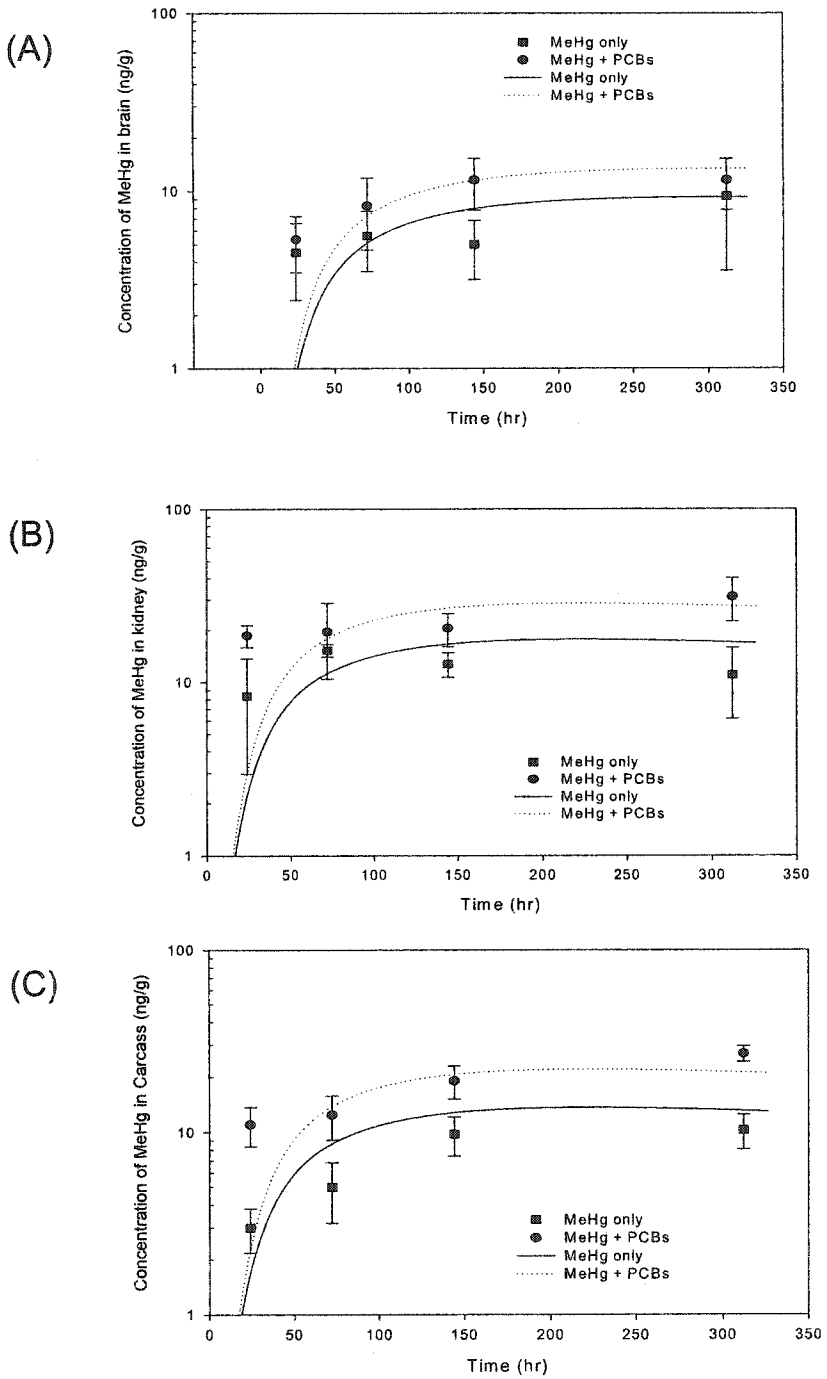

Fig. 4.5. PBPK simulation of MeHg in the pup's tissues comparing the group exposed to MeHg only with the group exposed to MeHg + PCB congeners. (A) Brain (B) Kidney (C) Carcass

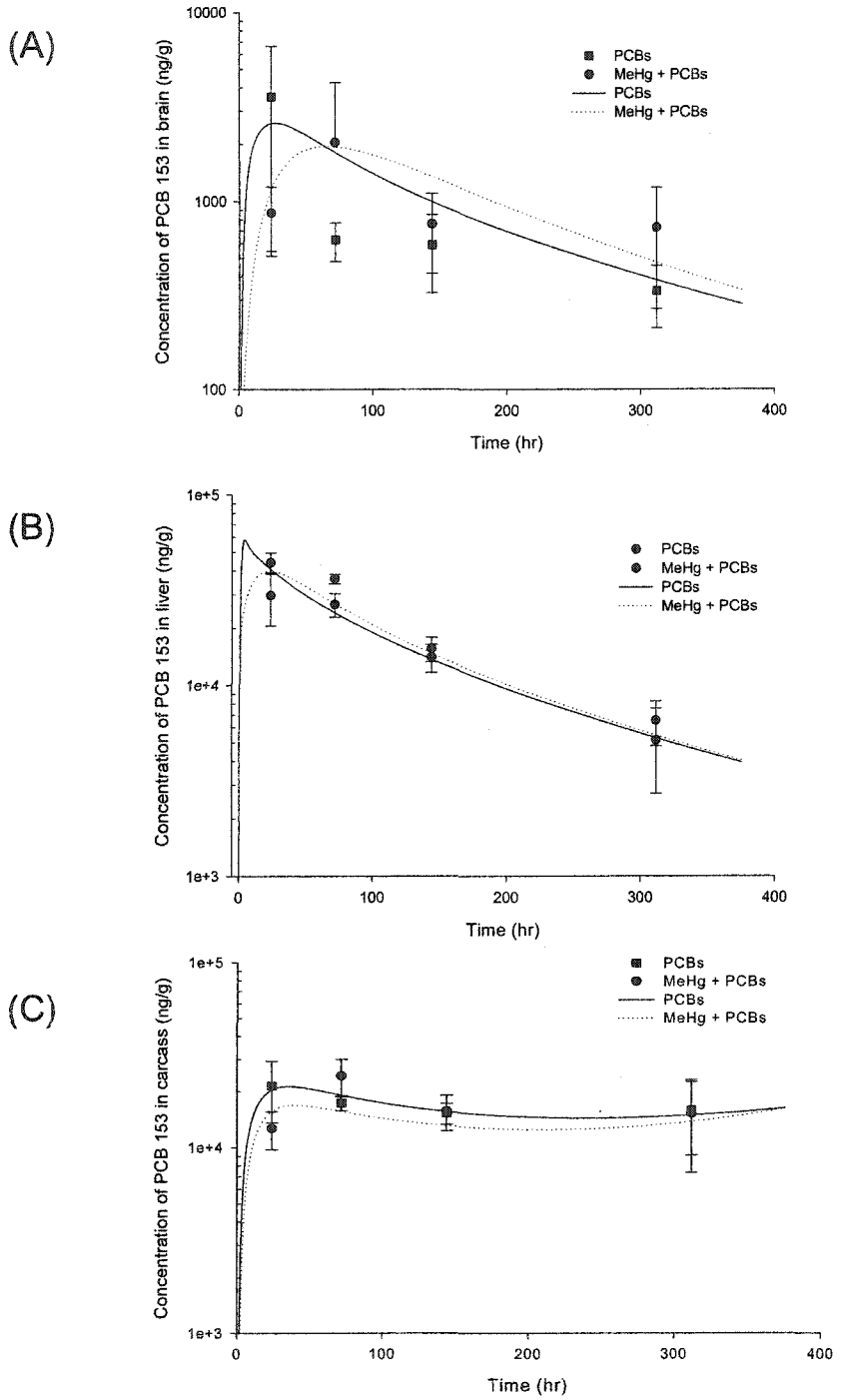

Fig. 4.6. PBPK simulation of PCB 153 in the pup's tissues comparing the group exposed to MeHg only with the group exposed to MeHg + PCB congeners. (A) Brain (B) Liver (C) Carcass

DISCUSSION

Comparison of lactational transfer and tissue dispositon of MeHg and PCB congeners between single chemical exposure and chemical mixture exposure.

The present study demonstrated that co-exposure to MeHg and PCB congeners in lactating mice could elicit pharmacokinetic interactions for the lactational transfer from the mother to the pups. In our study, the interactions were conspicuous for MeHg transfer. Overall, experimental results suggested that co-exposure with PCB congeners could increase the lactational transfer of MeHg to the pups. It has been shown that MeHg could be transferred from the mother to the babies during the perinatal period (Pitkin *et al.*, 1976). Most of these previous studies have focused on MeHg transfer during the gestational period (Risher *et al.*, 2002). Some animal studies have suggested that lactational transfer of MeHg may be less efficient than gestational transfer (Nordenhall *et al.*, 1995). However, it is still important to know the pharmacokinetic profiles on the lactational transfer and tissue disposition of MeHg as this chemical can induce neurotoxicities during the developmental period and important brain development occurs during the postnatal period. It has been extensively discussed that the developing brain is more sensitive to environmental contaminants than adult brain due to rapid growth during a short time period (Eriksson, 1997). Therefore, even slight changes of the concentrations of toxicants in the developing brain can elicit substantial damage and functional deficits. As mentioned above, we demonstrated that co-exposure with PCB congeners could increase the lactational transfer of MeHg. These findings suggest a need to perform

other studies involving pharmacokinetic interactions between environmental contaminants. In addition to PCB congeners which we selected, other environmental toxicants coexist with MeHg, especially in the water (Ritter *et al.*, 2002). Since most risk assessments are based on the single chemical studies, research on pharmacokinetic interactions will ultimately contribute to risk assessment for environmental contaminants.

Importance of transport proteins for pharmacokinetic interactions between MeHg and PCB congeners. Previous studies showed that MeHg or PCB congeners could affect the levels of albumin or lipoproteins in blood respectively (Borlakoglu *et al.*, 1990; Lachapelle *et al.*, 1993; Borlak and Thum, 2002). One of our goals for this study is to present mechanistic bases for pharmacokinetic interactions between MeHg and PCB congeners. It is well known that MeHg could bind to albumin with high affinity and PCB congeners could bind to lipoproteins (Matthews *et al.*, 1977; Yasutake *et al.*, 1989). In addition, lactational transfer of MeHg or PCB congeners with transport proteins has been suggested previously (Spindler-Vomachka *et al.*, 1984; Sundberg *et al.*, 1999). Thus, we hypothesized that lactational transfer of MeHg or PCB congeners will be changed if either of these chemicals could affect the levels of respective transport proteins (i.e., albumin or lipoproteins) in maternal blood. To investigate the hypothesis, we pursed two approaches as described in previous sections. We incorporated transport protein pools to explore the effects of transport protein levels on the lactational transfer of MeHg and PCB congeners. Then, we simulated tissue dispositions of the chemicals according to the levels of transport proteins. Simulation results showed different tissue dispositions depending on the

hypotheses. In all cases, the lactational transfer of MeHg or PCB congeners were affected by the changes of transport proteins. Only differences were the relationships between the amount of chemical transfer and the level of transport protein. Through PBPK simulation, we arrived at the conclusion that the experimental studies analyzing the relationships between the amount of chemical transfer and the level of transport protein in maternal blood will be helpful in revealing the mechanisms of pharmacokinetic interactions between MeHg and PCB congeners. Finally, experimental findings showed that the amount of MeHg transfer to the pups and the level of albumin in maternal blood were proportional. This result supported the hypothesis that only MeHg bound with albumin could be transported to the pups through milk. In addition, this result may explain the mechanisms of pharmacokinetic interactions between MeHg and PCB congeners as exposure to MeHg decreased the levels of albumin in maternal blood and co-exposure with PCB congeners compensated these effects of MeHg.

PBPK modeling for pharmacokinetic interactions. One of the advantages of PBPK modeling is the potential for predicting pharmacokinetic interactions between chemicals or drugs (Sugita *et al.*, 1982). Some PBPK studies have been performed to reveal pharmacokinetic interactions between toxic chemicals (el-Masri *et al.*, 1997). These studies mostly incorporated mechanistic information revealed by previous biological studies into their models to predict or validate changes in tissue disposition by pharmacokinetic interactions (Kanamitsu *et al.*, 2000). Our PBPK study is unique in terms that we used PBPK modeling to raise a reasonable hypothesis which could be validated by experimental approaches and further PBPK studies. This point is

important because we are exposed to many different chemical mixtures in real environment, which mostly we don't know mechanistic information on the interactive effects. It is clear that biological studies revealing mechanisms of interactive effects should be performed before we could predict pharmacokinetic interactions in complex chemical mixtures. Even though many studies are still going to reveal mechanisms of interactive effects, it is not always easy to do those works only based on biological knowledge. Our study explored the possibility that PBPK modeling will lead to reasonable hypothesis relating to the mechanisms of chemical interactions, followed by validation through experimental and modeling approaches. We believe that computational modeling and experimentation can play synergistic roles in both mechanistic studies and risk assessment. In conclusion, the modeling approach presented here would be useful for predicting the pharmacokinetics of chemical mixtures and revealing possible interaction mechanisms.

Future directions. Our pharmacokinetic research focused on revealing the disposition and mechanisms of MeHg and PCB congeners when lactating mice and their pups were exposed to the chemicals through a single exposure. We did not try to explore the pharmacokinetics of multiple dosing scenarios. Even though the pharmacokinetics of single dosing is useful in many ways regarding tissue dosimetry, pharmacokinetic interactions/their mechanisms, and construction/validation of PBPK modeling, it is necessary to perform pharmacokinetic studies under a multiple dosing scenario to get more comprehensive information. Thus, the pharmacokinetics of multiple dosing will be needed to investigate more extensive results.

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CHAPTER 5

Differential Expression of Cerebellar proteins in the Developing Brain of Mice Exposed to PCB Congeners and Methylmercury through Perinatal Transfer: a Proteomics Approach

Sun Ku Lee, John T. Belisle, and Raymond S.H. Yang

ABSTRACT

To explore the effects of PCB congeners and MeHg either alone or in combination on neurodevelopment, the expression profiles of proteins were analyzed in cerebellums of mice exposed to the chemicals during the perinatal period. From GD 7 until PND 21, each group of pregnant C57BL/6 mice were exposed to control, PCB 153 alone, PCB 126 alone, MeHg alone, a mixture of PCB 153 + PCB 126, a mixture of PCB 153 + MeHg, and a mixture of all three chemicals. This experimental design was to specifically evaluate the possible interactive neurodevelopmental effects of PCB 153 with co-exposure of PCB 126 and/or MeHg. At PND 9 and PND 30, cerebellums of neonatal mice were collected and total proteins were extracted. Two-dimensional electrophoreses were performed and the densities of protein spots were compared between control and chemical-treated groups. The protein spots statistically different from control were collected and extracted by in-gel digestion. The extracted protein spots were identified by tandem mass spectrometry coupled to liquid chromatography. The differentially expressed proteins were diverse including

structural, glycolysis-related, Ca^{++}/calmodulin signal transduction-related, energy balance-related, growth related and stress-related proteins. Further western blot analyses showed that other Ca^{++} or Ca^{++}/calmodulin signal transduction-related proteins were affected by chemical treatment. The expression patterns of proteins were different between single chemical treatment and chemical mixture treatment. These results suggest that perinatal exposure to PCBs and MeHg disrupts the expression profiles of proteins and such pharmacodynamic interactions between PCBs and MeHg may have important toxicological implications in perinatal neurodevelopment.

INTRODUCTION

The developing brain is different from adult brain in both composition and function. For example, the cholinergic effect and synaptic plasticity undergo a striking transformation within the first three weeks after birth in rats (Schomerus *et al.,* 1999). Also, there is a functional change in GABAergic actions from excitatory to inhibitory over the first two weeks of the postnatal period (Rich and Lichtman, 1989). The developing brain is more sensitive to environmental hazards than the adult brain (Eriksson and Talts, 2000). Exposure to environmental toxicants coincident with the ontogeny of the developmental process is more likely to cause adverse effects if they interfere with the cascade of these developmental processes (Weiss, 2000).

As described in previous chapters, PCBs and MeHg are ubiquitous environmental contaminants and are recognized as representative neurotoxicants (Myers *et al.,* 2000). In particular, developing brain is highly susceptible to both

toxicants (Boersma and Lanting, 2000). Epidemiological studies have revealed that the babies and children born at PCB and/or MeHg contaminated sites showed reduced cognitive function and disrupted neurobehavioral characteristics even if their mothers and adults did not show any indications of neurotoxicities (Mahaffey, 2000). Similar results were also obtained in several animal studies. For instance, the exposure to PCBs and/or MeHg could affect neurofunctional behaviors including sensory, motor system, and learning/memory function in rodents and primates (Seegal, 1996).

Recently, interactive effects between PCBs and MeHg have been shown in some studies. In an epidemiologic study, neurobehavioral deficits in children indicated a possible interaction between PCBs and MeHg, where PCBs could possibly augment the neurobehavioral deficits at increased levels of mercury exposure (Grandjean *et al.*, 2001). Bemis and Seegal showed that co-exposure to PCBs and MeHg affected cytosolic calcium homeostasis in a non-additive manner and reduced dopamine content in brain synergistically (Bemis and Seegal, 1999, 2000). These results raise the possibility that co-exposure of PCBs and MeHg through perinatal period may interactively affect normal brain development, resulting in functional deficits in babies and children.

Proteomics is the study of total protein expression in a defined biological system such as cellular genomes, cells, tissues, and organs. Proteomics can be a primary and robust approach to explore the expression changes of all possible proteins and to relate the expression changes in an integrated manner to functional deficits caused by toxicant exposure (Kennedy, 2002). In the present study, we have investigated the effects of co-exposure of PCB congeners and MeHg on developing

brain through perinatal transfer. In particular, we analyzed whether co-exposure to PCB congeners and MeHg affects the expression of proteins involved in intracellular and metabolic function of brain using two-dimensional electrophoresis and tandem mass spectrometry in addition to western blot analyses.

Our experimental design involved the co-exposure two different PCB congeners (i.e., PCB 153 and PCB 126) of and MeHg to pregnant mice during the perinatal period and analyzed the expression patterns of proteins in cerebellum of neonatal mice on PND 9 and 30. We selected PCB 153 and PCB 126 as representatives of PCB congeners. PCB 153 is a non-planar congener that appears prevalently in environment and mammalian tissues at the highest concentration (Muhlebach *et al.*, 1991). PCB 126, on the other hand, is the most toxic PCB congener with coplanar structure (Safe, 1994). Both congeners have been suggested to induce neurobehavioral deficits through perinatal exposure (Hussain *et al.*, 2000). We focused our attention on cerebellum for a variety of reasons. Cerebellum plays an important role in modulating motor commands and integrating sensory information (Hansel and Linden, 2000). It has been suggested that cerebellum is necessary for certain types of motor learning (Kim and Thompson, 1997) and is important for procedural memory, which is needed to use previously learned skills (Okano *et al.*, 2000). Cerebellum is very sensitive to the exposure of PCBs and MeHg. In MeHg exposure, granule and purkinje cells in cerebellum were damaged (Warfvinge, 2000). In PCB exposure, cytotoxicity and interruption of calcium homeostasis of granule cells in cerebellum have been already reported (Mundy *et al.*, 1999).

MATERIALS AND METHODS

Materials. PCB 153 and PCB 126 were obtained from Accustandard (New Haven, CT). MeHg and β-mercaptoethanol were purchased from Sigma-Aldrich Chemical (St. Louis, MO). Cell lysis buffer was obtained from Promega (Madison, WI). IPG strips were purchased from Amersham Biosciences (Piscataway, NJ). BCA protein assay kit was provided by Pierce Biotechnology (Rockford, IL). All other materials used in two-dimensional electrophoresis and western blot analyses were obtained from Bio-Rad Laboratories (Richmond, CA).

Animals. C57 BL/6 male and female mice were purchased from Harlan Sprague Dawley Laboratory (Indianapolis, IN) and housed at Painter Animal Center, Colorado State University. The mice were maintained on a 12-hr light/dark cycle at a constant temperature of 25 °C and humidity of 55%.

Mating and breeding. Mating took place at Painter Center, Colorado State University according to the following breeding protocol. Briefly, two female mice were placed together with one male in a cage for 4 consecutive days. We assigned the first day of cohabitation as GD 0. The pregnancy rate was approximately 60%. The animals were weighed every three days during gestation. On PND 0, 7, and 14, the offspring were counted and inspected for signs of overt toxicity.

Chemical treatments. Before mating, female mice were assigned randomly to seven experimental groups, each containing 10 individuals. After mating (i.e., first day of cohabitation), chemical treatments of all animals were initiated on GD 7. Each group of mice was treated everyday to control (vehicle only), PCB 153 alone (5 mg/kg/day), PCB 126 alone (5 *u*g/kg/day), MeHg alone (40 *u*g/kg/day), PCB 153 +

PCB 126, PCB 153 + MeHg, and PCB 153 + PCB 126 + MeHg. The exposure of chemicals started on GD 7 and ended on PND 21 when the pups were weaned. PCBs were administered in the diet and MeHg was administered via drinking water. The dosing concentrations were determined assuming that a female mouse consumes 5 g of water and 10 g of diet daily.

Brain sample preparation. On PND 9 and 30, the pups in each group were lightly anesthetized using isoflurane and immediately decapitated. In cold HBSS solution, cerebellum was isolated and minced with surgical scissors to enhance the homogenizing process. After discarding the dissection medium, 1 ml of cold cell lysis buffer was added to the samples and the samples were homogenized by a Teflon homogenizer followed by ultrasonication. Cell homogenates were centrifuged at 10,000 g for 20 minutes at 4 °C. Supernatants were collected and 1 ml of extraction solution was added to the pellets. Supernatants were collected one more time after homogenization and centrifugation of resuspended pellets. Spin dialysis was performed to remove salts, detergents, and insoluble materials. After resuspended with double distilled water, total protein concentration of each sample was determined by BCA protein assay.

Protein separation by two-dimensional electrophoresis. A 500 *u*g of protein sample was applied to IPG strip (pH 3-10 NL, 13 cm, Amersham Biosciences, Piscataway, NJ) on IPGphor (Amersham Biosciences, Piscataway, NJ) for isoelectric focusing. The IPG strip was hydrated for 16 hrs at 50 V and then focused gradually. After isoelectric focusing, the IPG strip was applied to a 12% SDS-polyacrylamide gel and proteins were separated by their mass. Gels were stained with Coomassie

Brilliant Blue R-250 staining solution. Stained gels were scanned and analyzed using PDQuest (Bio-Rad, Richmond, CA). All gels of each group were normalized to the same total integrated intensity of all spots and spot densities were compared between control and chemical treated groups.

In-gel digestion and tandem mass spectrometry. The selected spots were cut into small pieces and digested using modified trypsin (Boehringer Mannheim, Manheim, Germany). The digested peptides were extracted from gel matrices using 60% acetonitrile, 0.1% TFA. The final sample extracts were eluted onto liquid chromatography coupled to tandem mass spectrometer (MS/MS) after dissolved in an eluting solution (5% acetonitrile, 0.1% TFA). The peptide fragments were analyzed by MS and MS/MS. MS and MS/MS data of peptides were matched to mouse protein database (NCBI, http: www.ncbi.nlm.nih.gov/genome/guide/mouse/) using SEQUEST software.

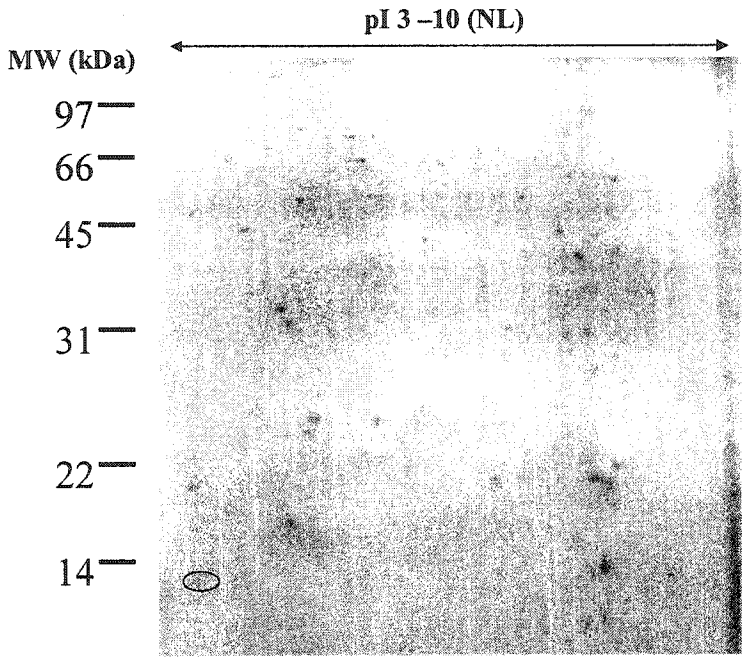
Western blot analyses. Western blot analyses were performed according to the instruction of immune-blot kit (Bio-Rad, Richmond, CA) with slight modifications. A 30 *u*g sample of total proteins was separated using electrophoresis and transferred onto nitrocellulose membranes by electrophoretic transfer. Membrane blots were incubated with antibodies directed against specific proteins (*i.e.*, MAPK, PKC, CaMK II, and CaMK IV) respectively, followed by the incubation with biotinylated goat anti-rabbit antibody. Proteins were detected with AP color development agents. The densities of detected proteins were quantitated using Alpha-Imager 2200(Alpha Innotech Corp., CA).

Statistical analyses. Differences between control and chemical-treated groups were tested for significance by student t-test. All analyses were performed with the statistical software, Minitab ($P < 0.05$; Windows version 12.0).

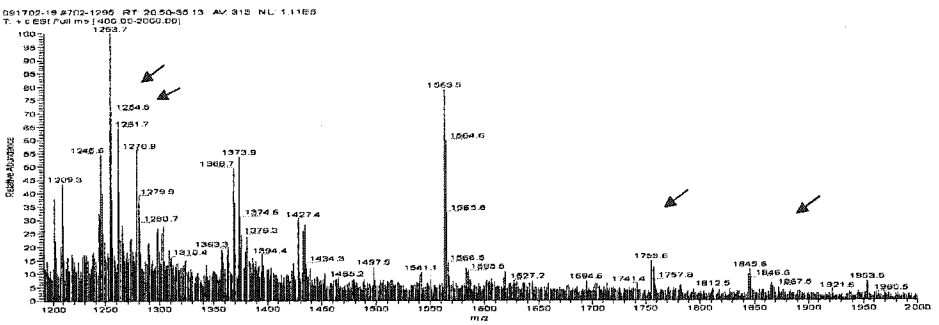
RESULTS

Quantitation and identification of differentially expressed proteins through perinatal transfer of MeHg and PCBs. A two-dimensional gel image was shown in Fig. 5.1. A. Approximately 300 protein spots were demonstrated clearly within an isolectric point (pI) range of 3.0 – 10.00 and molecular mass (MW) range of 10 – 100 kDa. Normalized spot densities were compared between control and each chemical-treated group using PDQuest. Initially, protein spots were selected for identification on the basis of a 1.5-fold or greater difference from control at each experiment. After three independent experiments, the spots whose densitites were statistically different from control were finally selected for identification and analyzed by tandem mass spectrometry. Fig. 5.1. B showed the representative mass spectrum of precursor ions obtained by the analysis of a selected protein spot. All abundant precursor ions were further fragmented into the product ions. The product ion spectra were converted to the theoretical peptide sequences and searched for matches in protein database. Fig. 5.1. C showed the identified protein of a selected spot. In this analysis, four different peptides were matched in one protein and approximately 40% were recovered from the whole protein sequence. To confirm whether the identification is correct, we checked pI and MW of the identified protein against literature values. Other protein spots were identified in a similar manner.

A



B



C

database=C:\Xcalibur\database\mousepepNCBI.fasta, accession=gi|20820398|ref|XP_133199.1|
peptide(s)=DGNGYISAAELR VFDKDGNGYISAAELR EAFSLFDKDGDGTITTK
EADIDGDGQVNYEEFVQMMTAK Analyzing ..

>gi|20820398|ref|XP_133199.1| calmodulin 3 [Mus musculus]
MADQLTEEQI AEFKEAFSLF DKDGDGTITT KELGTVMRSL GQNPTEAELQ DMINEVDADG
NGTIDFPEFL TMMARKMKDT DSEEEIREAF RVFDKDGNGY ISAAELRHVM TNLGEKLTDE
EVDEMIREAD IDGDGQVNYE EFVQMMTAK
>average mass = 16819 , pI = 4.09
position sequence (NCBI BLAST link) -------- --------
96- 107 DGNGYISAAELR
92- 107 VFDKDGNGYISAAELR
15- 31 EAFSLFDKDGDGTITTK
128- 149 EADIDGDGQVNYEEFVQMMTAK
Protein Coverage: 55/149 = 36.9% by amino acid count, 6036/16819 = 35.9% by mass

Fig. 5.1. Representative example of protein identification by two-dimensional electrophoresis and tandem mass spectrometry. (A) Gel image by two-dimensional electrophoresis. (B) Protein identification by tandem mass spectrometry. (C) Search result by Sequest.

The identified proteins were presented in Appendix. Detailed information of identified proteins including annotations, expression changes, and functions were also provided in Appendix.

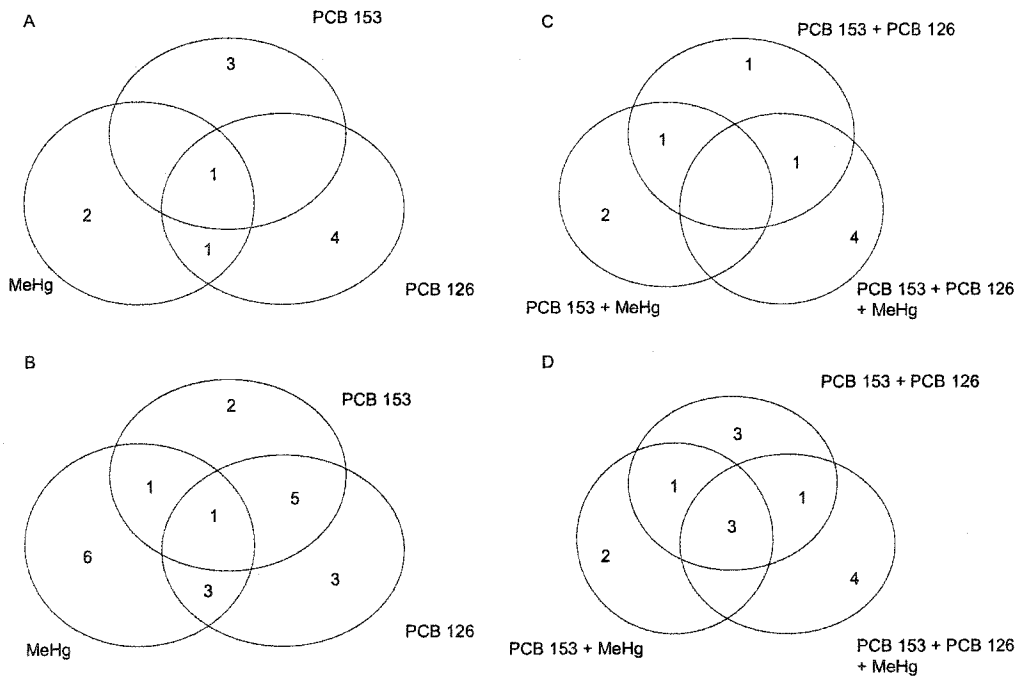


Fig. 5.2. Comparison of protein numbers affected by the exposure to MeHg and/or PCB congeners at PND 9. (A) proteins increased by single chemical treatment. (B) proteins decreased by single chemical treatment. (C) proteins increased by chemical mixture treatment. (D) proteins decreased by chemical mixture treatment.

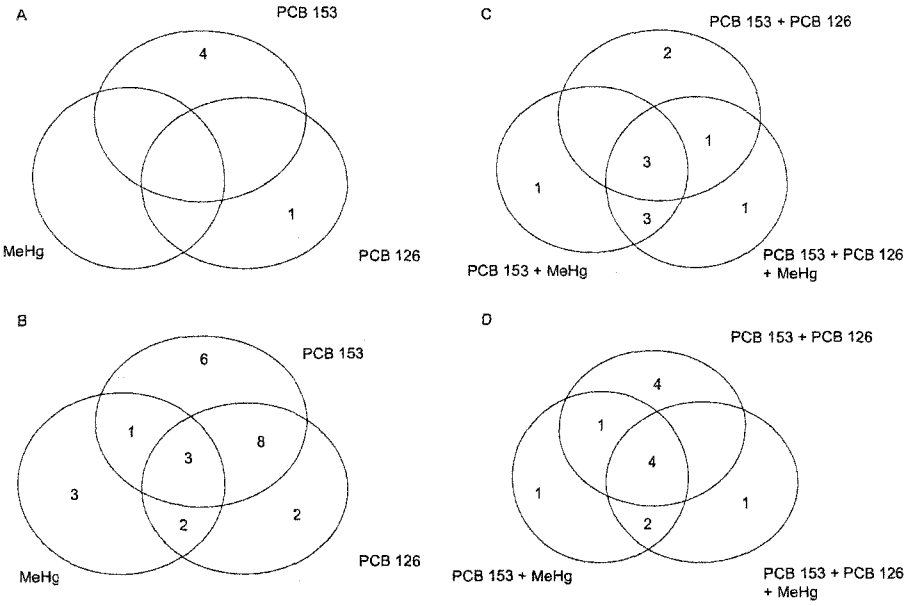


Fig. 5.3. Comparison of protein numbers affected by the exposure to MeHg and/or PCB congeners at PND 30. (A) proteins increased by single chemical treatment. (B) proteins decreased by single chemical treatment. (C) proteins increased by chemical mixture treatment. (D) proteins decreased by chemical mixture treatment.

Western blot analyses of additional proteins. Functional analyses of the above proteins suggested that some proteins are directly or indirectly connected to Ca^{++} or Ca^{++}/calmodulin related signal transduction pathway. Thus, western blot analyses were performed to determine whether perinatal exposure to PCB congeners and/or MeHg could affect the expression of proteins involved in Ca^{++} or Ca^{++}/calmodulin related signaling. The selected proteins were PKC, MAPK (=ERK ½), CaMK II, and CaMK IV, which are all important in brain function and involved in Ca^{++} or Ca^{++}/calmodulin related pathways. Fig. 5.4. showed the expression changes of selected cerebellum proteins affected by PCBs and/or MeHg on PND 9. Fig. 5.5. showed the expression changes of selected cerebellum proteins affected by MeHg

and/or PCBs on PND 30. Co-exposure of PCB congeners and MeHg induced interactive effects deviating from additivity.

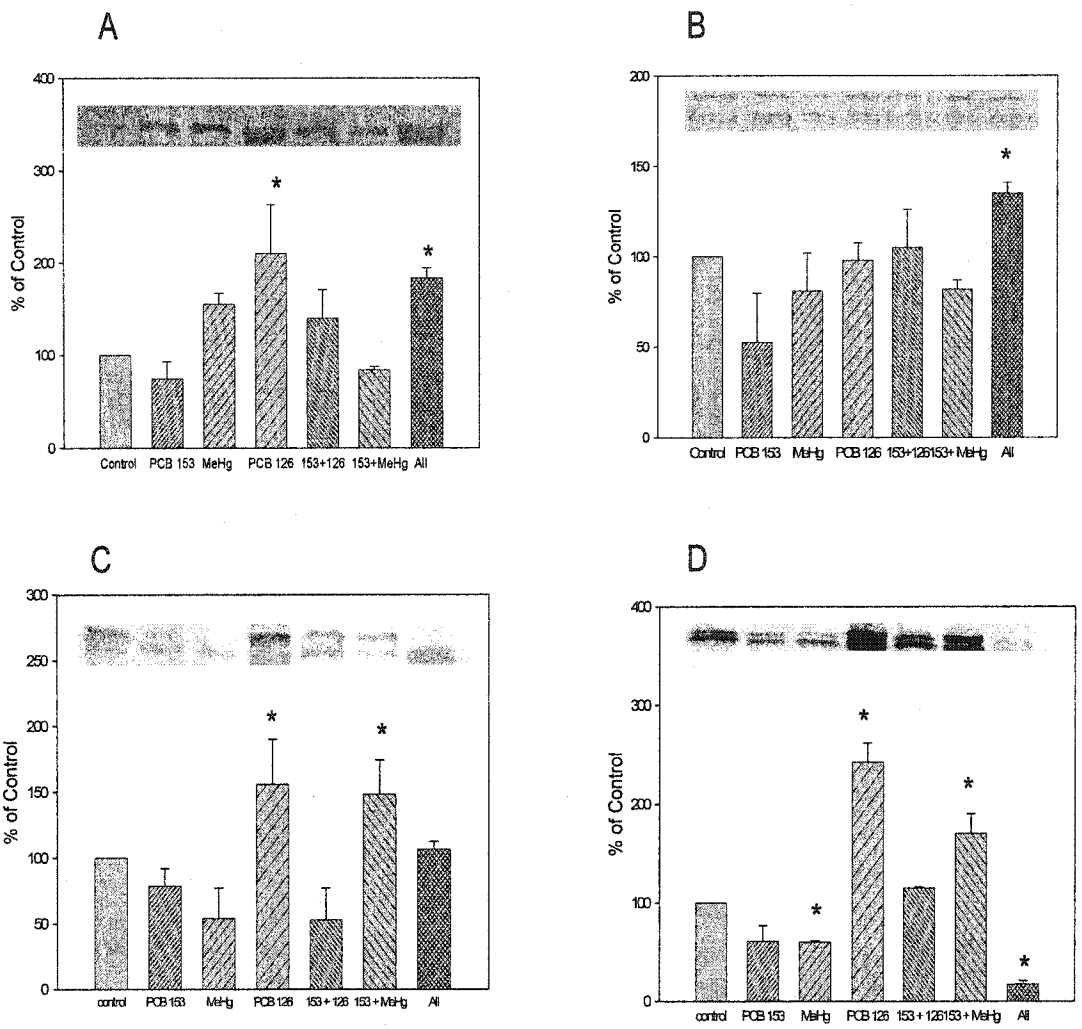


Fig. 5.4. Effects of MeHg and/or PCB congeners on selected protein expressions as measured by western blot analyses (At PND 9). An inset is the representative result of western blots. (A) CaMK IV expression. (B) PKC expression. (C) CaMK II expression. (D) MAPK expression.

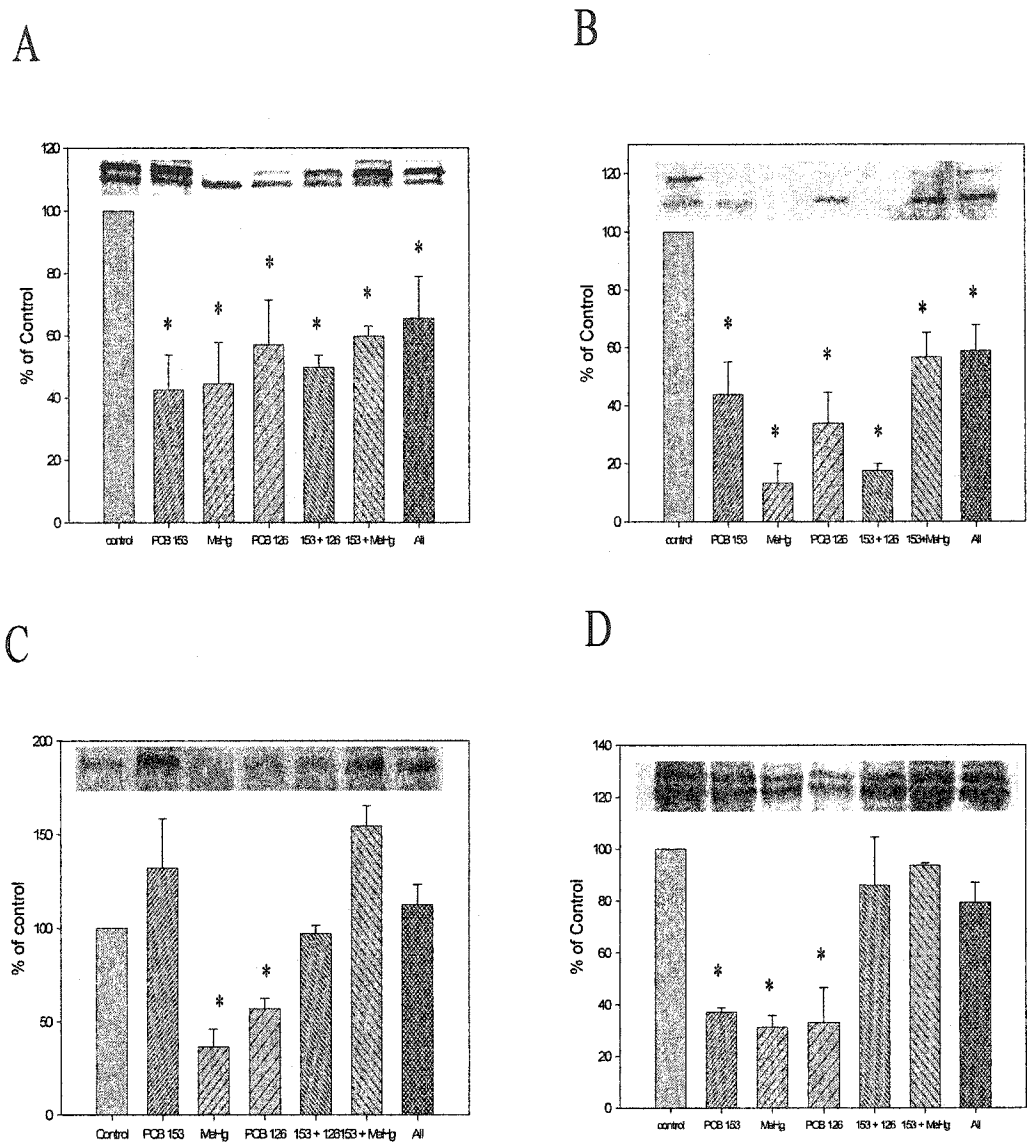


Fig. 5.5. Effects of MeHg and/or PCB congeners on selected protein expressions as measured by western blot analyses (At PND 30). An inset is the representative result of western blots. (A) CaMK IV expression. (B) PKC expression. (C) CaMK II expression. (D) MAPK expression.

Interactive effects of co-exposure to PCB congeners and MeHg. The expression pattern of affected proteins was much different between the groups exposed to single chemical and the groups exposed to chemical mixtures. To analyze this phenomenon further, we summarized the effects of co-exposure in Table 5.2 and 5.3. We categorized the identified proteins into three sections according to the exposure: the proteins affected both by single chemical exposure and chemical mixture exposure, the proteins affected only by single chemical exposure, and the proteins affected only by chemical mixture exposure. The number of proteins affected only by single chemical exposure or affected only by chemical mixture exposure exceeded the number of proteins affected both by single chemical exposure and chemical mixture exposure.

DISCUSSION

Effects of MeHg and PCB congeners on the expression of functional proteins. In our study, the expressions of many cerebellum proteins were affected by perinatal exposure to MeHg and/or PCB congeners. The characteristics of affected proteins were diverse and most of them are important in brain function. The most affected proteins were related to glucose or carbohydrate metabolism. The importance of glucose in brain function has been known for a long time. Glucose is the major energy source for the brain and is essential for maintaining normal brain function such as survival, learning, and memory (Messier and Gagonon, 1996). It has been suggested that some types of neurological disorders are associated with the impairment of glycolysis (Hovda *et al.*, 1995).

The proteins involved in stress-related response were also affected by chemical treatments. Since stress response is the basic adaptive process for cell survival, it is regarded that the affected proteins in this category may play roles in self-defense or adaptation against chemical challenge (Kopecek *et al.*, 2001).

Interruption of Ca^{++} and/or Ca^{++}/calmodulin signaling pathways by MeHg and PCB congeners. In addition to the diverse functional perspectives, one of the significant characteristics is that many proteins are involved directly or indirectly in Ca^{++} or Ca^{++}/calmodulin related signal transduction. Intracellular calcium plays an important role in brain function. In particular, it conveys signals involving the control of neurodevelopment, cellular functions, and death of neurons (Brini and Carafoli, 2000). In our results, the proteins regulating Ca^{++} homeostasis are creatine kinase, peptidylprolyl isomerase, and Cbl-b. Creatine kinase contributes to the maintenance of Ca^{++} homeostasis through the efficient delivery of ATP (de Groof *et al.*, 2002). Peptidylprolyl isomerase A can stabilize Ca^{++} channels (Schiene-Fischer and Yu, 2001). Cbl-b positively regulates receptor-mediated Ca^{++} signaling (Yasuda *et al.*, 2002).

Several proteins whose functions are regulated by Ca^{++} were also identified. Calmodulin 3 is a ubiquitous cytoplasmic calcium ion receptor (Ikura *et al.*, 2002). When cytosolic Ca^{++} increases, the regulatory subunits of phosphorylase kinase lose their function, leading to the induction of phosphotransferase activity (Rice *et al.*, 2002). Cofilin can be activated by dephosphorylation reactions initiated either by a GTP-binding protein or Ca^{++} (Davidson and Haslam 1994). The remodeling of actin structure requires the spatiotemporal regulation of Ca^{++} (Furuyashiki *et al.*, 2002).

ATP synthase is inhibited by Ca^{++}, resulting in the disruption of oxidative phosphorylation in intact mitochondria (Fagian *et al.*, 1986).

Some of the proteins are regulated by Ca^{++} related proteins, not directly by Ca^{++}. Pyruvate kinase can be activated by CaMK II (Mieskes *et al.*, 1987). KCIP-1 is a potent inhibitor of PKC (Toker *et al.*, 1992). The expression of diazepam binding inhibitor is mediated by CaMK II activation (Katsura *et al.*, 2000).

Western blot analyses also supported that Ca^{++} or Ca^{++}/CaM related proteins were affected by chemical treatments. CaMK IV has been implicated in nuclear signaling events that control neuronal growth, differentiation, and plasticity when it is activated by the increase in cytosolic Ca^{++} (Ahn *et al.*, 1999). MAPK (=ERK ½) is activated by CaM-binding proteins as Ras-GRF and CaMK IV. During development, MAPK signaling pathway promotes synaptic connectivity, neurite outgrowth, and cell survival in cerebellum (Mao *et al.*, 1999). Phosphorylation of CaMK II by cytosolic calcium plays an important role in the growth of neurites and the development of neuronal complexity (Borodinsky *et al.*, 2002). PKC can be directly activated by cytosolic Ca^{++} and plays a role in synaptic plasticity (Daniel *et al.*, 1998).

Interactive effects of co-exposure to PCB congeners and MeHg. Our results suggested that there are interactive effects on protein expression between PCB congeners and MeHg. First, the net effects of PCB congeners and/or MeHg on protein expression were different between single chemical exposure and chemical mixture exposure. Second, the expression patterns of affected proteins were different between single chemical exposure and chemical mixture exposure. At this stage, it is difficult to know the precise mechanisms of interactive effects. However, based on

our finding so far, the following two hypotheses for interactive effects may be proposed. First, pharmacokinetic interactions between PCB congeners and MeHg affect the perinatal transfer of each individual chemical. In this scenario, the chemical concentration in the brain region may be different between single chemical exposure and chemical mixture exposure. Work is in progress to test this hypothesis. Second, interactive effects on Ca^{++} regulation will induce protein expression changes in the brain. The above discussion involving Ca^{++} signaling interruption is reminiscent of the result of Bemis *et al* (2000). In that article, they showed that co-exposure of PCBs and MeHg induced synergistic effects at low dose exposure and antagonistic effect at high dose exposure on the increase of intracellular Ca^{++} concentration. The functions of many proteins are dependent upon intracellular Ca^{++} levels (Brini and Carafoli, 2000). Therefore, interactive effects between MeHg and PCB congeners could change the effects of each respective chemical.

Time-dependent changes of affected proteins. Regarding time-dependent changes, we found three distinct characteristics: First, certain cerebellum protein expression levels (peptidylprolyl isomerase, phosphorylase kinase, and MAPK) were decreased by chemical treatments both at PND 9 and 30. Interestingly, these proteins play roles in cellular growth. Thus, this effect may be related to the inhibition of growth and development by PCBs and MeHg (Grandjean *et al.,* 2003). Second, some cerebellum protein expression levels (thioredoxin peroxidase, and CaMK IV) were induced first and inhibited later or vice versa. It seems possible that this pattern may be caused by the compensation mechanism maintaining homeostasis (Radice *et al.,* 1998). Third, most cerebellum protein expression levels shown in Tables and Figures

were affected only at PND 9 or PND 30. This suggests that proteins may have different sensitivities to chemical exposure during developmental period.

In conclusion, we showed that many functional proteins were affected by perinatal exposure of PCB congeners and MeHg. We also suggested that co-exposure to PCB congeners and MeHg can induce interactive effects on protein expressions. Further studies are needed for linking protein expressions with functional deficits.

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Table 5.1. Comparison of protein expression between single chemical exposure and chemical mixture exposure.

Time of assay	Chemical exposure	Number of proteins affected by chemical exposure	
		Increase	Decrease
PND 9	Single chemical	11	21
	Chemical mixture	11	15
PND 30	Single chemical	6	25
	Chemical mixture	18	7

Table 5.2. Interactive effects of co-exposure of MeHg and PCB congeners on the protein expression (At PND 9)

Protein expression	Chemical treatment		
	single		Chemical mixture
	153	126	153+126
increase	Matrix protein M1 Phospholipase C, alpha Tubulin alpha 6	ATP synthase, beta Actin beta Brain lipid biding protein Cytochrome C oxidase polypeptide Tubulin alpha 6	Cofilin Thioredoxin peroxidase 1
decrease	Enolase alpha Phosphoglycerate dehydrogenase Peptidylprolyl isomerase Phosphorylase kinase Thioredoxin peroxidase 1	Alpha chain precursor Enolase alpha Phosphoglycerate dehydrogenase Eukaryotic translation elongation factor 1 Kringle-containing transmembrane protein 1 Phosphoglycerate mutase 1 Phosphorylase kinase Tubulin T beta 15	ATP synthase DnaK-type molecular chaperone Actin gamma Glucose regulated protein 78kDa Transcription regulator, ArcR Tubulin alpha 6 Voltage dependent anion channel 1
	153	MeHg	153+MeHg
increase	Matrix protein M1 Phospholipase C, alpha Tubulin alpha 6	Dihydropyrimidinase like 2 Tubulin alpha 6 Ubiquitin carboxyterminal hydrolase L1	Glucose regulated protein 78kDa Thioredoxin peroxidase 1 3-oxoacid CoA transferase transforming growth factor beta 2 Tubulin T beta 15
decrease	Enolase alpha Phosphoglycerate dehydrogenase Peptidylprolyl isomerase Phosphorylase kinase Thioredoxin peroxidase 1	Alpha chain precursor Eukaryotic translation elongation factor 1 Peptidylprolyl isomerase A Synuclein beta Tropomyosin 2 beta Tryptophan hydroxylase	Coffilin Transcription regulator ArcR Tubulin alpha 6 Voltage dependent anion channel 1

	Voltage dependent anion channel 1		
	153+126	MeHg	153+126+MeHg
increase	Cofilin Thioredoxin peroxidase 1	Dihydropyrimidinase like 2 Tubulin alpha 6 Ubiquitin carboxyterminal hydrolase L1	Eukaryotic translaton elongation factor 1 Phosphoglycerate mutase T-complex protein 1 Vimentin
decrease	ATP synthase DnaK-type molecular chaperone Actin gamma Glucose regulated protein 78kDa Transcription regulator, ArcR Tubulin alpha 6 Voltage dependent anion channel 1	Alpha chain precursor Eukaryotic translation elongation factor 1 Peptidylprolyl isomerase A Synuclein beta Tropomyosin 2 beta Tryptophan hydroxylase Voltage dependent anion channel 1	ATP synthase Heat shock protein 60 kDa Polyamine oxidaseprobable inhibitor protein Transcription regulator ArcR Voltage dependent anion channel 1

Table 5.3. Interactive effects of co-exposure of MeHg and PCB congeners on the protein expression (At PND 30)

Protein expression	Chemical treatment		
	single	Chemical mixture	
	153	126	153+126
increase	Actin 2 alpha ATP synthase beta Collagen alpha 2 Glucose regulated protein 78 kDa Thioredoxin peroxidase 1 Ubiquitin carboxyterminal hydrolase L1	KCIP-1	Carbonic anhydrase 2 Enolase alpha Peptidylprolyl isomerase A Protocadherin beta Ubiquitin/ribosomal L40
decrease	Aldolase 1 Calmodulin 3 Cbl-b Cofillin Peptidylprolyl isomerase A Phosphoglycerate kinase 1 Phosphorylase kinase Pyruvate kinase Syntaxin 2 Triosephosphate isomerase Zebrin 2	Aldolase 1 ATP synthase alpha Calmodulin 3 Creatine kinase Peptidylprolyl isomerase A Phosphoglycerate kinase 1 Phosphorylase kinase Pyruvate kinase Syntaxin 2 Zebrin 2	Heat shock protein 70 kDa Syntaxin 2
	153	MeHg	153+MeHg
increase	Actin alpha 2 ATP synthase beta Collagen alpha 2 Glucose regulated protein 78 kDa Thioredoxin peroxidase 1 Ubiquitin carboxyterminal hydrolase L1		ATP synthase beta Actin beta Diazepam binding inhibitor DnaK type molecular chaperon Heat shock protein 70 kDa Peptidylprolyl isomerase A Testis derived transcript Zinc finger 1A2

decrease	Aldolase 1 Calmodulin 3 Cbl-b Cofillin Peptidylprolyl isomerase A Phosphoglycerate kinase 1 Phosphorylase kinase Pyruvate kinase Syntaxin 2 Triosephosphate isomerase Zebrin 2	Actin alpha 2 Actin beta Calmodulin 3 Creatine kinase Phosphorylase kinase Zebrin 2 Zincfinger 1A4	Enolase 2 Syntaxin 2
	153+126	MeHg	153+126+MeHg
increase	Carbonic anhydrase 2 Enolase alpha Peptidylprolyl isomerase A Protocadherin beta Ubiquitin/ribosomal L40		Alpha glucosidase Actin beta Creatine kinase Diazepam binding inhibitor Enolase alpha Peptidylprolyl isomerase A Protocadherin beta RNA helicase Ubiquitin/ribosomal L40 Zinc finger 1A2
decrease	Heat shock protein 70 kDa Syntaxin 2	Actin alpha 2 Actin beta Calmodulin 3 Creatine kinase Phosphorylase kinase Zebrin 2 Zincfinger 1A4	Syntaxin 2

Appendix 5.1. Identification of proteins significantly affected by single chemical exposure (At PND 9).

Spot No.	NCBI accession No.	Protein identification[a]	Amino acid sequence coverage (%)[b]	Possible roles[d]	Expression change (fold by control)[e]		
					PCB 153	PCB 126	MeHg
1	763156	Alpha chain precursor	51.0	Immune function	0.5±0.5	0.2±0.3	0.3±0.5
2	20898164	Alpha-enolase	9.5	Glycolysis	0.3±0.4	0.1±0.2	0.5±0.5
3	25052136	ATP synthase beta	13.2	Energy balance	0.8±1.0	2.4±2.2	1.4±1.3
4	20908535	AW048587	5.8		0.2±0.1	9.0±11	3.0±1.0
5	4501885	Beta actin	31.5	Structural integrity	0.7±0.4	1.8±0.6	0.6±0.5
6	20859575	Brain lipid-binding protein	43.6	Neuronal differentiation	5.4±8.2	4.7±3.5	2.0±1.1
7	20865514	CG4393-PA	5.3	Signal transduction	1.0±0.5	1.1±0.2	0.3±0.3
8	116982	Cytochrome C oxidase polypeptide	6.0	Energy balance	0.9±0.5	0.7±0.2	0.8±0.9
9	6753676	Dihydropyrimidinase-like 2	26.9	Neuronal growth	1.3±0.5	0.9±0.5	1.4±0.4
10	28488414	D-3-phosphoglycerate dehydrogenase	4.8	Glycolysis	0.3±0.5	0.4±0.5	1.4±0.6
11	9055210	Eukaryotic translation elongation factor 1	20.9	Transcriptional regulation	0.6±0.5	0.5±0.4	0.3±0.3
12	14161700	kringle-containing transmembrane protein 1	7.0	Signal transduction	0.8±0.7	0.7±0.3	1.3±0.5
13	77190	Matrix protein M1	11.9		2.7±1.6	0.7±0.6	2.0±2.8

14	20853425	Peptidylprolyl isomerase A	32.3	Acceleration of protein folding	0.6±1.0	0.7±0.5	1.0±0.8
15	6679291	Phosphoglycerate mutase 1	11.8	Glycolysis	0.3±0.5	0.7±0.2	0.7±0.4
16	6679687	Phospholipase C, alpha	26.9	Signal transduction	1.6±0.6	1.2±0.3	0.8±0.8
17	6755054	Phosphorylase kinase	36.9	Glycogenolysis	1.1±0.7	0.6±0.1	0.7±0.4
18	15809030	Synuclein, beta	15.4	Neuronal plasticity	0.9±1.4	0.6±0.7	0.4±0.3
19	20885677	Thioredoxin peroxidase 1	17.2	Antioxidant	0.5±0.2	1.1±0.2	0.8±1.0
20	20826465	Tropomyosin 2, beta	6.3	Structural integrity	0.7±0.5	0.8±0.6	0.4±0.4
21	6678411	Tryptophan hydroxylase	10.5	Serotonin synthesis	0.6±0.5	3.9±6.0	0.1±0.1
22	20844376	Tubulin alpha 6	22.3	Structural integrity	1.9±0.8	2.3±1.1	2.3±0.9
23	20844824	Tubulin T beta15	10.5	Structural integrity	1.1±0.9	0.6±0.4	0.5±0.5
24	20831193	Ubiquitin carboxy-terminal hydrolase L1	40.4	deubiquitination	1.1±0.4	1.5±0.8	1.2±0.2
25	20880458	Voltage-dependent anion channel 1	23.3	Energy balance	0.8±0.4	0.8±0.3	0.8±0.2
26	20828714	Hypothetical protein[c]	12.5		0.6±0.5	0.4±0.5	2.2±3.2
27	20869836	Hypothetical protein	16.8		0.7±0.1	0.6±0.1	0.6±0.9

28	26328207	Unnamed protein	12.0	0.3±0.9	0.9±0.3	0.6±0.3

[a]Protein names are listed as the NCBI database.
[b]The portion of the protein containing matched peptide fragments from MS/MS analysis.
[c]Hypothetical protein is the protein sequence predicted from NCBI contig by automated computational analysis using gene prediction method.
[d]Information were obtained from SWISS-PROT database.
[e]Values are represented as mean±S.D.

Appendix 5.2. Identification of proteins significantly affected by chemical mixture exposure (At PND 9).

Spot No.	NCBI accession No.	Protein identification[a]	Amino acid sequence coverage (%)[b]	Possible roles[d]	Expression change (fold by control)[e]		
					PCB 153 + PCB 126	PCB 153 + MeHg	PCB 153 + PCB 126 + MeHg
1	6680748	ATP synthase	15.7	Energy balance	0.5±0.3	0.8±0.8	0.6±0.3
2	20884735	Cofilin	27.1	Control of anctin polymerization	2.0±0.6	0.6±0.4	1.0±1.1
3	27716941	DnaK-type molecular chaperone	18.0	Molecular chaperon	0.4±0.3	1.1±0.6	0.9±0.5
4	9055210	Eukaryotic translation elongation factor 1 beta	20.9	Transcriptional regulation	1.7±1.9	8.3±13	4.2±3.6
5	20840713	Gamma-actin	4.8	Structural integrity	0.6±0.3	1.5±1.1	1.5±0.9
6	1304157	Glucose regulated protein, 78 kD	7.5	Signal transduction	0.5±0.4	1.7±0.7	1.4±0.5
7	20846982	HSP 60	8.9	Molecular chaperon	0.9±0.8	1.7±1.2	0.5±0.4
8	20864145	Phosphoglycerate mutase	11.8	Glycolysis	1.6±0.7	1.3±0.4	1.7±0.2
9	21704050	Polyamine oxidase 1	2.2	Amino acid metabolism	0.6±0.6	1.2±1.0	0.5±0.4
10	7513784	Probable inhibitor protein	3.0	Signal transduction	0.6±0.4	1.0±0.5	0.1±0.1
11	20913657	RIKEN cDNA 0610009D10	47.2		0.1	0.3±0.1	0.3±0.5
12	22654291	T-complex protein 1	31.0	Molecular chaperon	1.1±1.1	0.8±0.7	2.1±0.5

13	20885677	Thioredoxin peroxidase 1	17.2	Antioxidant	1.4±0.4	1.8±0.5	2.1±1.8
14	18266680	3-oxoacid CoA transferase	9.2	Lipid metabolism	0.9±0.4	1.6±0.3	0.8±0.4
15	6678317	Transforming growth factor, beta 2	5.0	Cell growth	0.9±0.4	1.5±0.4	1.1±0.4
16	16120226	Transcription regulator, ArcR	25.0	Transcriptional regulation	0.3±0.2	0.4±0.2	0.5±0.3
17	20844376	Tubulin alpha-6	12.8	Structural integrity	0.5±0.4	0.2±0.1	1.1±0.6
18	20844824	Tubulin T beta15	10.5	Structural integrity	3.0±2.3	1.2±0.1	1.5±1.7
19	2078001	Vimentin	18.0	Structural integrity	0.9±0.6	0.8±0.5	4.0±1.2
20	20880458	Voltage-dependent anion channel 1	23.3	Energy balance	0.5±0.4	0.7±0.2	0.7±0.3
21	20883628	Hypothetical protein	3.9		1.3±0.9	0.6±0.7	0.3±0.3
22	26324710	Unnamed protein	4.0		1.0±0.2	0.7±0.2	0.4±0.4

[a]Protein names are listed as the NCBI database.
[b]The portion of the protein containing matched peptide fragments from MS/MS analysis.
[c]Hypothetical protein is the protein sequence predicted from NCBI contig by automated computational analysis using gene prediction method.
[d]Information were obtained from SWISS-PROT database.
[e] Values are represented as mean±S.D.

Appendix 5.3. Identification of proteins significantly affected by single chemical treatment (At PND 30).

Spot No.	NCBI accession No.	Protein identification[a]	Amino acid sequence coverage (%)[b]	Possible roles[d]	Expression change (fold by control)[e]		
					PCB 153	PCB 126	MeHg
1	6671539	aldolase 1	9.8	carbohydrate metabolism	0.2±0.3	0.3±0.4	0.5±0.6
2	4501883	alpha 2 actin	15.4	cell motility	2.5±0.8	1.0±0.5	0.5±0.4
3	6680748	ATP synthase alpha	15.7	energy balance	0.9±0.6	0.3±0.2	0.5±0.6
4	20455479	ATP synthase, beta	12.5	ATP synthesis	1.2±0.2	1.4±1.0	0.9±0.9
5	4501885	beta actin	31.5	cell motility	5.0±4.0	1.2±0.6	0.3±0.3
6	20820398	calmodulin 3	36.9	signal transduction	0.2±0.2	0	0.1±0.2
7	25065860	Cbl-b	4.3	signal transduction	0.6±0.1	0.2±0.2	0.5±0.6
8	20884735	cofilin	27.1	control of actin polymerization	0.4±0.5	1.0±1.3	0.5±0.6
9	1083212	collagen a2	5.0	forming of the fibril	2.4±0.6	0.7±0.5	1.0±0.5
10	20859147	creatine kinase	8.1	energy transduction	0.9±0.6	0.4±0.2	0.3±0.5
11	11612489	glucose regulated protein, 78 kDa	4.9	signal tranduction	8.1±5.6	0.6±0.4	1.3±1.2
12	20898720	KCIP-1	7.3	signal tranduction	0.6±0.9	24±10	1.2±0.6
13	20873906	MOR106-4	6.5		0.7±0.2	1.3±1.2	2.0±1.7
14	20853425	Peptidylprolyl isomerase A	32.3	acceleration of protein folding	0.7±0.2	0.5±0.2	0.5±0.5
15	6679291	phosphoglycerate kinase 1	25.4	glycolysis	0.5±0.1	0.2±0.2	0.5±0.4

16	6755054	phosphorylase kinase	36.9	glycogenolysis	0.6±0.4	0.4±0.5	0.4±0.4
17	1363219	pyruvate kinase	10.9	glycolysis	0.4±0.1	0.3±0.2	0.4±0.6
18	6679667	syntaxin 2	14.2	epithelial morphogenesis	0.1±0.1	0.1±0.1	0.5±0.6
19	6755758	thioredoxin peroxidase 1	17.2	antioxidant	1.4±0.3	40±1.0	1.2±1.1
20	6678413	triosephosphate isomerase	18.1	glucose metabolism	0.7±0.3	1.0±0.9	1.1±0.7
21	20831193	ubiquitin carboxy-terminal hydrolase L1	9.4	de-ubiquitination	1.3±0.2	1.7±1.7	2.7±2.3
22	2118269	zebrin 2	39.7	glycolysis	0.6±0.2	0.4±0.1	0.7±0.2
23	20867772	zinc finger 1A4	3.9	transcriptional regulation	1.8±1.5	1.0±0.7	0.5±0.2
24	20821527	hypothetical protein[c]	10.8		0.8±0.1	3.5±5.3	2.9±3.7
25	20910025	hypothetical protein	17.0		0.8±0.1	1.9±2.0	0.8±0.5
26	20882687	hypothetical protein	10.4		0.2±0.2	0.6±0.9	0.5±0.4
27	20914974	hypothetical protein	6.8		1.1±0.2	0.4±0.2	0.8±0.4
28	20883126	hypothetical protein	9.5		0.6±0.4	0.9±0.7	1.1±0.9

29	20869869	hypothetical protein	9.8	1.2±1.1	0.5±0.3	0.8±0.5

[a]Protein names are listed as the NCBI database.
[b]The portion of the protein containing matched peptide fragments from MS/MS analysis.
[c]Hypothetical protein is the protein sequence predicted from NCBI contig by automated computational analysis using gene prediction method.
[d]Information were obtained from SWISS-PROT database.
[e]Values are represented as mean±S.D.

Appendix 5.4. Identification of proteins significantly affected by chemical mixture treatment (At PND 30).

Spot No.	NCBI accession No.	Protein identification[a]	Amino acid sequence coverage (%)[b]	Possible roles[d]	Expression change (fold by control)[e]		
					PCB 153 + PCB 126	PCB 153 + MeHg	PCB 153 + PCB 126 + MeHg
1	20882731	Aldolase 3	8.5		0.9±0.5	0.5±0.3	1.1±1.0
2	6679465	alpha glucosidase II	5.6	regulation of folded proteins	1.1±1.0	1.3±0.9	2.5±0.6
3	25052136	ATP synthase, beta	13.2	energy balance	6.3±9.0	4.0±3.0	4.5±6.0
4	4501885	beta actin	31.5	cell motility	1.8±1.4	3.0±1.9	2.2±0.5
5	19745181	carbonic anhydrase 2	8.5	maintenance of cytoplasmic pH	11±10	7.8±6.8	4.6±4.7
6	20859147	creatine kinase	8.1	energy transduction	1.8±2.0	1.9±1.4	1.8±0.5
7	20910017	diazepam binding inhibitor	39.1	modulation of GABA Rc.	19±32	17±15	15±12
8	25049322	dnaK type molecular chaperon	21.2	molecular chaperon	1.8±1.1	2.4±1.4	1.8±1.0
9	25056911	enolase alpha	15.4	glycolysis	2.2±0.7	1.2±0.7	2.5±1.0
10	7305027	enolase 2	18.7	glycolysis	0.9±0.3	0.7±0.2	1.2±1.2
11	13624307	HSP 70	12.4	molecular chaperon	0.5±0.1	1.4±0.1	1.0±0.3
12	6031172	MDM2	22.5	ubiquitin ligation	7.1±9.4	1.4±0.4	1.0±0.4
13	20853425	peptidyl prolyl isomerase A	32.3	acceleration of protein folding	4.3±0.5	4.2±0.5	5.4±0.7

14	20877746	protocadherin beta 7	4.5	cell adhesion	5.1±3.8	3.2±2.6	2.6±1.8
15	20858197	RIKEN cDNA B230210I21	6.9		0.7±0.2	0.8±0.3	0.7±0.4
16	20895542	RNA helicase	3.4	ribosomal processing	1.9±1.9	0.9±0.6	2.6±1.3
17	6679667	syntaxin 2	14.2	epithelial morphogenesis	0.4±0.4	0.3±0.2	0.3±0.4
18	6755769	testis derived transcript	6.6	gene regulation	9.5±14	4.3±0.8	6.9±9.6
19	20877602	ubiquitin/ ribosomal L40	13.1	ribosomal protein	5.9±4.7	5.6±5.1	22±16
20	7106455	zinc finger 1A2	8.7	transcriptional regulation	3.8±5.5	20±3.5	17±2.8
21	20910025	hypothetical protein[c]	17.0		0.5±0.1	0.8±0.2	0.5±0.4
22	23593194	hypothetical protein	5.4		0.3±0.2	1.0±0.3	0.5±0.4
23	20893786	hypothetical protein	4.0		1.1±0.4	1.6±0.5	1.1±0.8
24	20883126	hypothetical protein	9.5		3.6±0.4	1.5±0.7	2.6±3.1

[a]Protein names are listed as the NCBI database.
[b]The portion of the protein containing matched peptide fragments from MS/MS analysis.
[c]Hypothetical protein is the protein sequence predicted from NCBI contig by automated computational analysis using gene prediction method.
[d]Information were obtained from SWISS-PROT database.
[e] Values are represented as mean±S.D.

CHAPTER 6

Proteomic Analyses for Differential Expression of Hippocampal Proteins by Perinatal Exposure of PCB Congeners and MeHg

Sun Ku Lee, John T. Belisle, and Raymond S.H. Yang

ABSTRACT

To explore the effects of PCB congeners and MeHg either alone or in combination on neurodevelopment, the protein expression profiles were analyzed in hippocampi of mice exposed to the chemicals via perinatal transfer. From GD 7 until PND 21, each group of pregnant C57BL/6 mice were exposed to control, PCB 153 alone (5 mg/kg), PCB 126 alone (5 ug/kg), MeHg alone (40 ug/kg), a mixture of PCB 153 + PCB 126, a mixture of PCB 153 + MeHg, and a mixture of all three chemicals. This experimental design is to specifically evaluate the possible interactive neurodevelopmental effects of PCB 153 with co-exposure of PCB 126 and/or MeHg. At PND 9 and 30, hippocampi of neonatal mice were collected and proteins were extracted. Two-dimensional electrophoreses and tandem mass spectrometry were performed to identify the protein spots affected by chemical exposure. The identified proteins were diverse including structural, glycolysis related, Ca/CaM related, energy balance related, growth related, and stress related proteins. Some functional proteins important in neuronal activity were also identified. Further western blot analyses

showed that other Ca^{++} or Ca^{++}/calmodulin signal transduction-related proteins were affected by chemical treatment. The expression patterns of proteins were different between single chemical treatment and chemical mixture treatment. These results suggest that perinatal exposure of PCBs and MeHg disrupts the expression profiles of proteins involved in the important functional aspects of cellular metabolism. Pharmacokinetic and pharmacodynamic interactions between both types of chemicals could change the effects of each single chemical.

INTRODUCTION

Polychlorinated biphenyls (PCBs) and Methylmercury (MeHg)are ubiquitous environmental contaminants and recognized as representative neurotoxicants (Tilson and Kodavanti, 1998; Myers *et al.*, 2000). Especially, developing brain is highly susceptible to both toxicants because of rapid growth and development (Boersma and Lanting, 2000; Rice, 2000). Epidemiological studies have revealed that the babies and children born at PCB and/or MeHg contaminated sites showed reduced cognitive function and disrupted neurobehavioral characteristics even if their mothers and adults didn't show any indications of neurotoxicities (Buck, 1996; Mahaffey, 2000). These results were also confirmed in several animal studies (Rice, 1996; Seegal, 1996). The animal studies commonly suggested that perinatal exposure to PCBs and MeHg can be the cause of neurofunctional deficits including the interruption in learning and memory (Newland and Paletz, 2000).

Recently, interactive effects between PCBs and MeHg are shown in some studies. In an epidemiologic study, neurobehavioral deficits in children indicated a possible interaction between PCBs and MeHg when they are exposed to higher

concentration of PCBs and mercury through perinatal exposure (Grandjean *et al.*, 2001). Bemis and Seegal showed that co-exposure to PCBs and MeHg affects cytosolic calcium homeostasis in a non-additive manner and reduces dopamine content in brain synergistically (Bemis and Seegal, 1999; Bemis and Seegal, 2000). These results raise a possibility that co-exposure of PCBs and MeHg through perinatal period interactively affects normal brain development, resulting in functional deficits in babies and children.

Hippocampus plays an important role in learning and declarative memory. Declarative memory includes episodic memory (memory for particular episodes) and semantic memory (memory for the facts) (Rolls, 2000). Both PCBs and MeHg have been reported to affect learning and memory in humans and animals (Jacobson and Jacobson, 2002; Grandjean *et al.*, 2003). From mechanistic standpoints, PCB exposure can inhibit LTP, an important indicator for synaptic plasticity, and other synaptic transmissions (Gilbert and Liang, 1998; Hong *et al.*, 1998; Hussain *et al.*, 2000) whereas MeHg exposure induces the interruptions of synaptic transmission and electrical excitability in hippocampal neurons (Yuan and Atchison, 1997; Fountain and Rowan, 2000). In addition to the neurophysiological interruptions, many biochemical and molecular biological changes have been associated with PCB and/or MeHg exposure (Kuznetsov, 1990; Costa *et al.*, 2001). Thus, the investigation of mixture effects on neurodevelopment will be critically important in risk assessment.

Proteomics is the study investigating whole expression of total proteins in a defined biological system such as cellular genomes, cells, tissues, and organs. While the cellular genome is relatively stable, a proteome constantly adapts to changes in

the cellular environment. Proteomics can be the primary and robust approach to explore the expression changes of all possible proteins, integrate them and relate to functional deficits caused by toxicant exposure (Kennedy, 2002). The technologies used are two-dimensional electrophoresis and tandem mass spectrometry coupled with liquid chromatography. These are the key technologies for proteomics because they can separate complex protein mixtures and identify each protein components in an efficient manner.

To explore the effects of co-exposure of PCB congeners and MeHg during the perinatal period, we exposed two different PCB congeners (i.e., PCB 153 and PCB 126) and MeHg to pregnant mice during perinatal period (between GD 7 and PND 21) and analyzed the expression patterns of proteins in hippocampi of neonatal mice on PND 9 and 30. We selected PCB 153 and PCB 126 as a representative of PCBs. PCB 153 is a non-planar congener that appears in environment and mammalian tissues at the highest concentration (Muhlebach *et al.*, 1991). PCB 126 is the most toxic PCB congener with coplanar structure (Safe, 1994). Both congeners have been suggested to induce neurobehavioral deficits through perinatal exposure (Rice and Hayward, 1999; Hussain *et al.*, 2000). We selected PCB 153 as a lead toxic agent because of its high content in human tissues and its known neurotoxicities. Additionally, in an earlier study in our laboratory (Lee *et al.*, 2002), the results suggested that lactational transfer of PCB 153 was enhanced when co-exposed to PCB 126.

MATERIALS AND METHODS

Materials. PCB 153 and PCB 126 were obtained from Accustandard (New Haven, CT). MeHg and β-mercaptoethanol were purchased from Sigma-Aldrich Chemical (St. Louis, MO). The purities of chemicals used were over 98 %, which was confirmed by both vendors. HBSS was purchased from In Vitrogen (Grand Island, NY). Cell lysis buffer was obtained from Promega (Madison, WI). Anti-CaMK II, and anti-CaMK IV were provided from Calbiochem (San Diego, CA). IPG strips were purchased from Amersham Biosciences (Piscataway, NJ). BCA protein assay kit was provided by Pierce Biotechnology (Rockford, IL). All materials used in two-dimensional electrophoresis and western blot analyses including amplified AP immune-blot kit, nitrocellulose membrane, blot paper, electrophoresis gel, Laemli sample buffer, TBST (Tris buffered saline with Tween-20) buffer, running buffer, transfer buffer, and dry fat milk were obtained from Bio-Rad Laboratories (Richmond, CA).

Animals. C57 BL/6 male and female mice were purchased from Harlan Sprague Dawley Laboratory (Indianapolis, IN) and housed at Painter Animal Center, Colorado State University, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). The mice were maintained on a 12-hr light/dark cycle at a constant temperature of 25 °C and humidity of 55%. Diet (certified Teklad NIH-07 rodent diet) and tap water were provided *ad libitum*. Before mating, the mice were allowed to acclimatize for 2 weeks.

Mating and breeding. Mating took place at Painter Center, Colorado State University according to the following breeding protocol. Briefly, two female mice were placed together with one male per cage during 4 consecutive days. One day

after mating (i.e., the first day of cohabitation), we assigned that as GD 0. Afterwards, the animals were separated and two female mice were housed together. The pregnancy rate was about 60%. The animals were weighed every three days during gestation. If the weights of mice increased dramatically, they were considered to be pregnant and housed individually. On PND 0, 7, and 14, the offspring were counted and inspected for signs of overt toxicity. Litters were not culled but litters with less than 4 pups were not used in the experiments.

Chemical treatments. Before mating, the female mice were assigned randomly to seven experimental groups, each containing 10 individuals. After mating (i.e., the first day of cohabitation), chemical treatments of all animals were initiated on GD 7. Each group of mice was treated everyday to control (vehicle only), PCB 153 alone (5 mg/kg/day), PCB 126 alone (5 *u*g/kg/day), MeHg alone (50 *u*g/kg/day), PCB 153 + PCB 126, PCB 153 + MeHg, and PCB 153 + PCB 126 + MeHg. The exposure of chemicals started on GD 7 and ended on PND 21 when the pups were weaned. PCBs were treated through diet and MeHg was treated via drinking water. The chemical concentration was determined assuming that a female mouse consumes 5 g of water and 10 g of diet. Stock solution of MeHg (100 *u*g/L) was made and stored at 4 °C. The stock solution was diluted to the final concentration right before treatment. Food pellets containing PCBs were prepared as follows. Each PCB congeners were dissolved completely to 10 ml of acetone and these solutions were mixed with small amounts (100 g) of powder diets. The mixed diets were dried completely in the chemical hood and further mixed with large amounts of powder diets (6.8 kg) using a mixer provided by Hobart Corporation (Troy, OH). The diets

containing PCBs were stored at room temperature without light exposure. The equal distribution of each PCB congeners in diet was checked by the measurement of PCB concentration using gas chromatography.

Dissection of brain regions and sample preparation. On PND 9 and 30, the pups of each group were lightly anesthetized using isoflurane and immediately decapitated. The brains were rapidly removed, and rinsed with cold HBSS solution. In cold HBSS solution, the collected brains were dissected and hippocampus was obtained. Hippocampus was minced with surgical scissors to enhance homogenizing process. After discarding incubation medium, 1 ml of cold cell lysis buffer (62.5 mM Tris–HCl (pH 8.0), 25% glycerol, 2% sodium dodesyl sulfate, 0.01% bromophenol blue, 1% TritonX-100, 5 mM EDTA, 1% NP 40, 1 mM NaF, 1 mM Na_3VO_4, 0.5% protease inhibitor cocktail) was added to the samples and samples were homogenized by Teflon homogenizer followed by ultrasonication. Cell homogenates were centrifuged at 10,000 g for 20 minutes at 4 °C. Supernatants were collected and 1 ml of extraction solution (5 M urea, 2 M thiourea, 2 % CHAPS, 2 mM TBP, 40 mM Tris, 0.2 % ampholytes) was added to the pellets. Supernatants were collected after homogenization and centrifugation depicted above. Both supernatants were combined and spin dialysis was performed to remove salts, detergents, and insoluble materials. After resuspended with double distilled water, total protein concentration of each sample was determined by BCA protein assay. Each sample aliquot was dried under a vacuum centrifuge. Until use, all dried samples were stored at – 20 °C.

Protein separation by two-dimensional electrophoresis. Dried protein samples were solubilized in the rehydration buffer (8 M urea, 2% CHAPS, 50 mM

DTT, 0.5% 3-10 ampholyte, 0.001% bromophenol blue). Total 500 ug of protein sample was applied to IPG strip (pH 3-10 NL, 13 cm, Amersham Biosciences, Piscataway, NJ) on IPGphor (Amersham Biosciences, Piscataway, NJ) for isoelectric focusing. IPG strip was hydrated for 16 hrs at 50 V and then focused gradually as follows: 1 hr at 500 V (gradient), 1hr at 1000 V (step-N-hold), 1.5 hr at 4000 V (gradient), 1 hr at 4000 V (step-N-hold), 2 hr at 8000 V (gradient), 6 hr at 8000 V (step-N-hold). After isoelectric focusing, the IPG strip was equilibrated for 30 min in an equilibration buffer (50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, 50 mM DTT, trace amount of bromophenol, pH 8.8). The IPG strip was applied to 12% SDS-polyacrylamide gel and proteins were separated by their mass. Gels were stained with Coomassie Brilliant Blue R-250 staining solution (0.1% Coomassie Blue R-250 in 40% methanol, 10% acetic acid). Stained gels were scanned and analyzed using PDQuest (Bio-Rad, Richmond, CA). After digitizing images, all gels of each group were normalized to the same total integrated intensity of all spots and spot densities were compared between control and chemical treated groups. After performing three independent experiments, the spots, whose densities were statistically different from control, were selected for further analysis.

In-gel digestion and tandem mass spectrometry. The selected spots were cut into small pieces and digested using modified trypsin (Boehringer Mannheim, Manheim, Germany) for 16 hrs. The digested peptides were extracted from gel matrices using 60% acetonitrile, 0.1% TFA. The sample extracts were dried under vacuum centrifuge. The final sample extracts were eluted onto liquid chromatography coupled to tandem mass spectrometer after dissolved in an eluting

solution (5% acetonitrile, 0.1% TFA). The effluent was directly introduced into a Finnigan LCQ (Thermoquest, San Jose, CA) electrospray mass spectrometer and the peptide fragments were analyzed by MS and MS/MS. MS and MS/MS data of peptides were matched to mouse protein database (NCBI, http: www.ncbi.nlm.nih.gov/genome/guide/mouse/) using SEQUEST software.

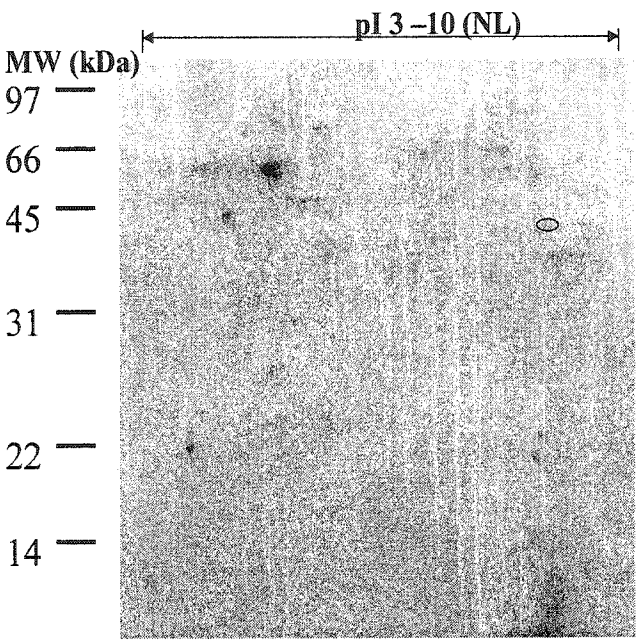
Western blot analyses. Western blot analyses were performed according to the instruction manual of immune-blot kit (Bio-Rad, Richmond, CA) with slight modifications. Briefly, a 30 *u*g of sample proteins was separated using electrophoresis on a 10% SDS-polyacrylamide gel under reducing conditions. Separated samples were further transferred onto nitrocellulose membranes by electrophoretic transfer. Membrane blots were incubated with antibodies (1:1000 dilution) directed against specific proteins (*i.e.*, PKC, CaMK II, and CaMK IV) respectively overnight with agitation. Membrane blots were incubated with biotinylated goat anti-rabbit antibody at a dilution of 1:2500 for 2 hr at room temperature. Following wash out, membrane blots were incubated with streptavin-biotinylated alkaline phosphatase (1:3000 dilution) for 2 hr at room temperature. Finally, proteins were detected with AP color development agents. The densities of detected proteins were quantitated using Alpha-Imager 2200(Alpha Innotech Corp., CA). The linear relationship between density and protein amount was confirmed using control samples whenever we tried to measure different proteins.

Statistical analyses. Differences between control and chemical-treated groups were tested for significance by student t-test. All analyses were performed with the statistical software, Minitab ($P < 0.05$; Windows version 12.0).

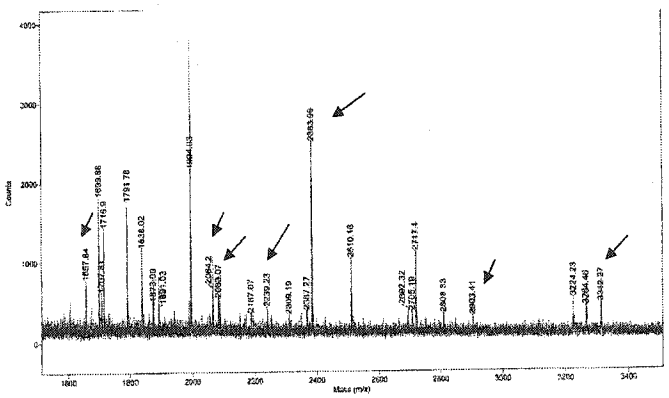
RESULTS

Protein identification and quantitation.

A



B



C

Reference: gi|6671539|ref|NP_031464.1| aldolase 1, A isoform [Mus musculus]
Database: mouse.fasta
Number of Amino Acids: 364 Monoisotopic Mass: 39332.3 pI: 8.30

Protein:

MPHPYPALTP EQKKELSDIA HRIVAPGKGI LAADESTGSI AKRLQSIGTE
NTEENRRFYR QLLLTADDRV NPCIGGVILF HETLYQKADD GRPFPQVIKS
KGGVVGIKVD KGVVPLAGTN GETTTQGLDG LSERCAQYKK DGADFAKWRC
VLKIGEHTPS ALAIMENANV LARYASICQQ NGIVPIVEPE ILPDGDHDLK
RCQYVTEKVL AAVYKALSDH HVYLEGTLLK PNMVTPGHAC TQKFSNEEIA
MATVTALRRT VPPAVTGVTF LSGGQSEEEA SINLNAINKC PLLKPWALTF
SYGRALQASA LKAWGGKKEN LKAAQEEYIK RALANSLACQ GKYTPSGQSG
AAASESLFIS NHAY

Protein Coverage:

Sequence	% Mass	AA	% AA
VNPCIGGVILFHETLYQK	5.16	70 - 87	4.95
GVVPLAGTNGETTTQGLDGLSER	5.76	112 - 134	6.32
IGENTPSALAIMENANVLAR	5.36	154 - 173	5.49
FSNEEIANGRVTALR	1.20	244 - 258	4.12
TVPPAVTGVTFLSGGQSEEEASINLNAINK	7.74	260 - 289	8.24
ALANSLACQGKYTPSGQSGAAASESLFISNHAY	8.43	332 - 364	9.07
YTPSGQSGAAASESLFISNHAY	5.74	343 - 364	6.04
Totals:	36.42	139	38.19

Fig. 6.1. Representative example of protein identification by two-dimensional electrophoresis and tandem mass spectrometry. A 500 *u*g protein of hippocampus (At PND 9) was separated by two-dimensional electrophoresis according to Materials and Methods.

(A) Gel image by two-dimensional electrophoresis.

(B) Protein identification by tandem mass spectrometry. The MS/MS spectrum was further analyzed by SEQUEST and searched public database as depicted in Methods. The arrows indicate the matched mass spectrum through database search.

(C) Search result by Sequest. There were significant matches of 7 peptide fragments in one protein. The result document is the partial summary of matched protein.

Figure 6.1 illustrates the typical pattern of two dimensional electrophoreses of hippocampal proteins in mice. Approximately 300 protein spots were demonstrated clearly within an isolectric point (pI) range of 3.0 – 10.00 and molecular mass (MW) range of 10 – 100 kDa. Normalized spot densities were compared between control and each chemical treated group using PDQuest. As described in Materials and Methods, the proteins spots whose densities were statistically different from control were identified by tandem mass spectrometry.

The results of protein identifications were presented in Appendix. Detailed information of identified proteins including annotations, expression changes, and functions were also provided in Appendix.

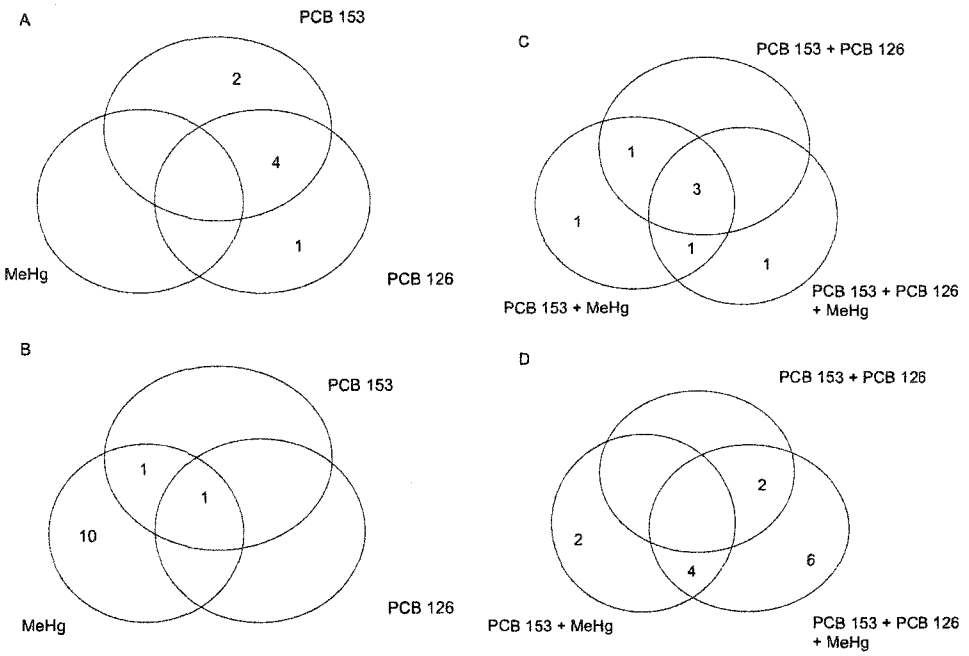


Fig. 6.2. Comparison of protein numbers affected by the exposure to MeHg and/or PCB congeners at PND 9. (A) proteins increased by single chemical treatment. (B) proteins decreased by single chemical treatment. (C) proteins increased by chemical mixture treatment. (D) proteins decreased by chemical mixture treatment.

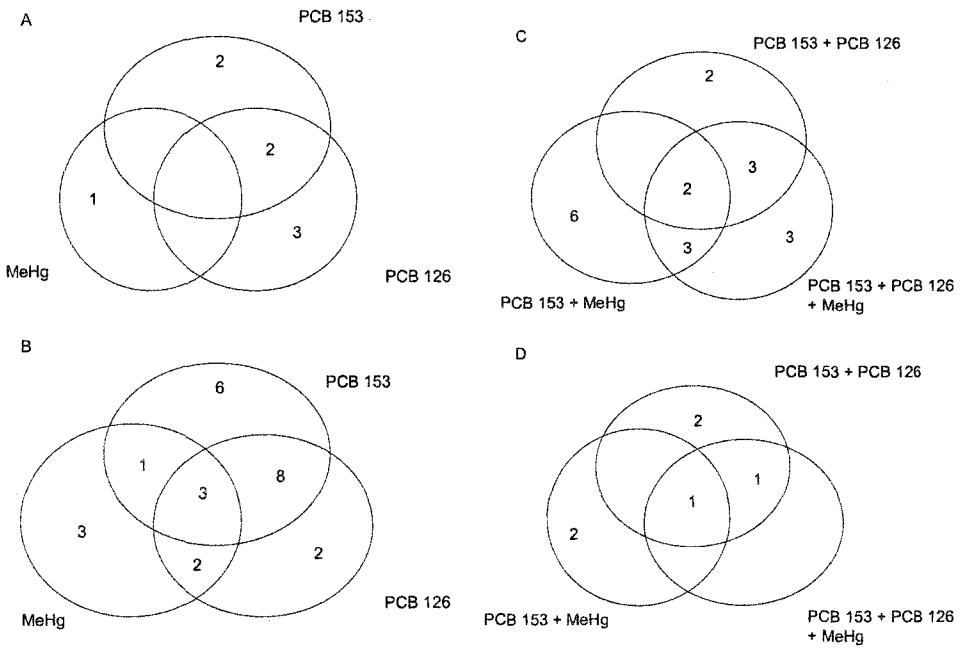


Fig. 6.3. Comparison of protein numbers affected by the exposure to MeHg and/or PCB congeners at PND 30. (A) proteins increased by single chemical treatment. (B) proteins decreased by single chemical treatment. (C) proteins increased by chemical mixture treatment. (D) proteins decreased by chemical mixture treatment.

Western blot analyses. Functional analyses of the identified proteins suggested that several proteins are directly or indirectly connected Ca^{++} or Ca^{++}/calmodulin related signal transduction pathway. Thus, western blot analyses were performed to determine whether perinatal exposure of PCB congeners and/or MeHg could affect the expression of proteins involved in Ca^{++} or Ca^{++}/calmodulin related signaling. The selected proteins were PKC, MAPK (=ERK ½), CaMK II, and CaMK IV, which are all important in brain function and involved in Ca^{++} or Ca^{++}/calmodulin related pathways. Fig. 6.2 showed the expression changes of each proteins affected by PCBs and/or MeHg at PND 9. The expressions of CaMK IV and CaMK II increased in the group exposed to the ternary mixture, MeHg + PCB 153 + PCB 126 increased comparing with control. Other groups did not show statistically significant increases. The expressions of PKC and MAPK decreased in the groups exposed to PCB 126 only, MeHg only, and PCB 153 + PCB 126. Other groups did not show any significant changes. Fig. 6.3 showed the expression changes of each proteins affected by PCBs and/or MeHg at PND 30. The expression of CaMK IV decreased in the groups exposed to PCB 153 only, PCB 126 only, and MeHg only. The expression of CaMK II decreased in the groups exposed to PCB 126 only, and MeHg only. The expression of PKC decreased in the groups exposed to PCB 153 + PCB 126 + MeHg, and MeHg only. The expression of MAPK decreased in the group exposed to MeHg

only. These results showed that perinatal exposure to MeHg and/or PCB congeners affected the expression of selected proteins in hippocampus. In addition, co-exposure of PCB congeners and MeHg induced interactive effects deviating from additivity.

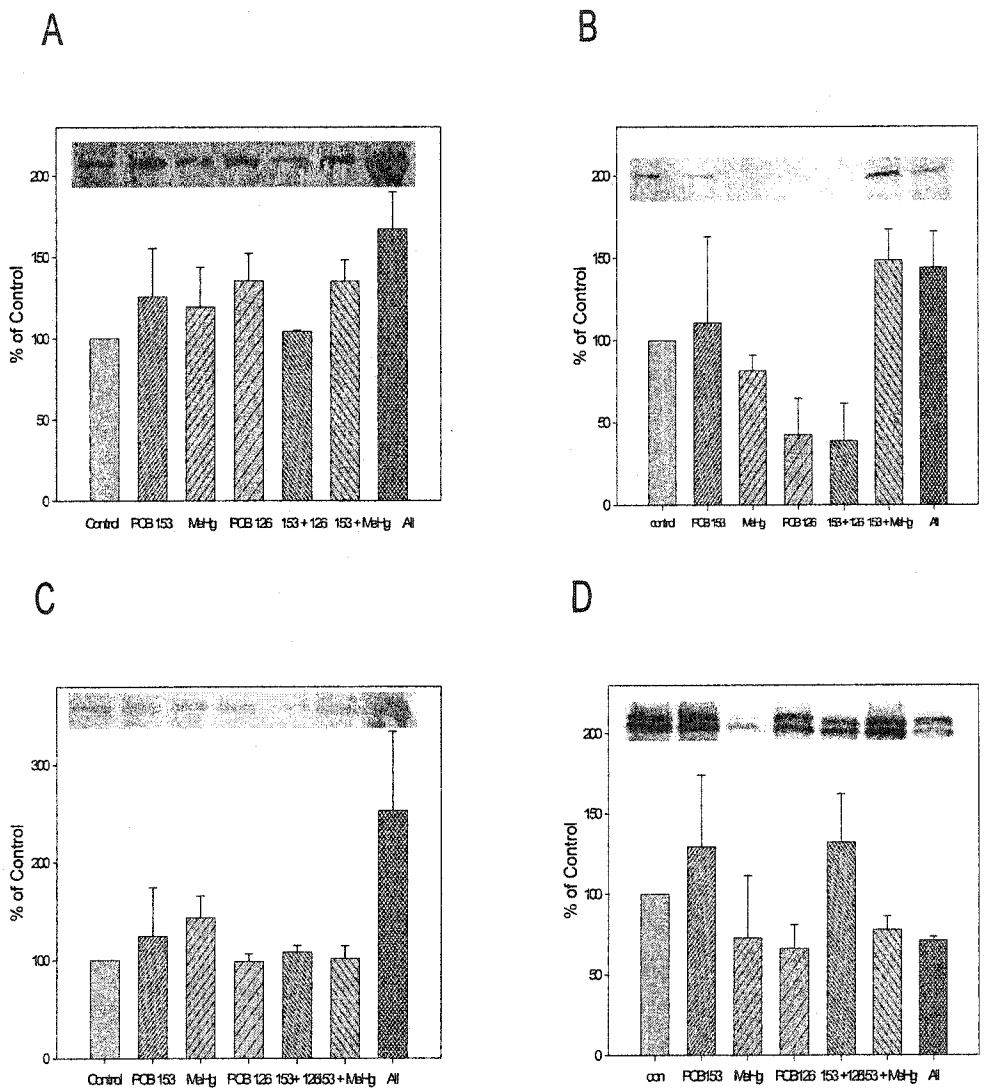


Fig. 6.4. Effects of MeHg and/or PCB congeners on selected protein expressions as measured by western blot analyses (At PND 9). An inset is the

representative result of western blots. (A) CaMK IV expression. (B) PKC expression. (C) CaMK II expression. (D) MAPK expression.

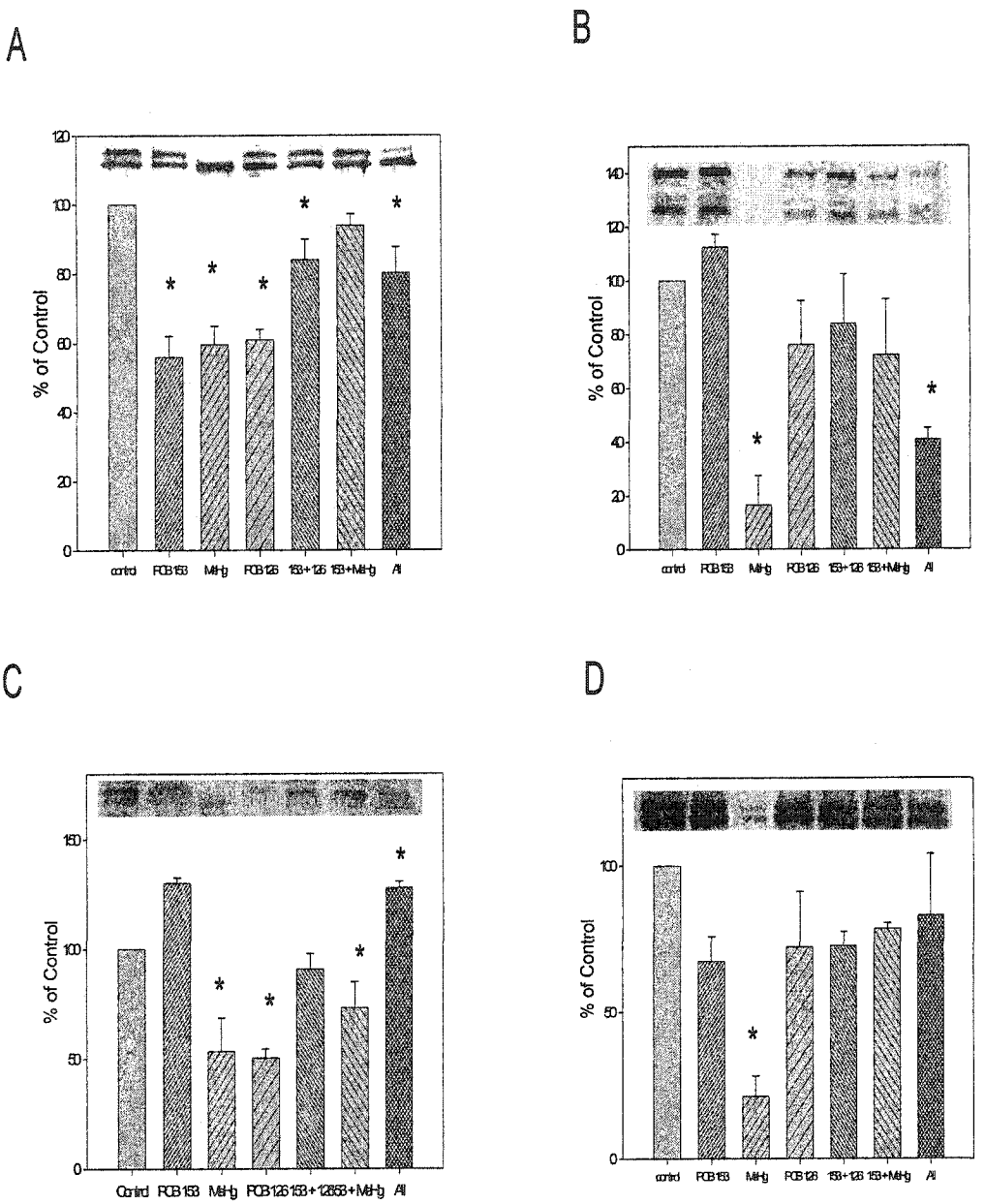


Fig. 6.5. Effects of MeHg and/or PCB congeners on selected protein expressions as measured by western blot analyses (At PND 30). An inset is

the representative result of western blots. (A) CaMK IV expression. (B) PKC expression. (C) CaMK II expression. (D) MAPK expression.

DISCUSSION

Our significant results can be summarized as follows. First, the proteomics investigation showed that perinatal exposure to MeHg and/or PCB congeners elicited differential expression of functional proteins in hippocampus of mice. Second, the differentially expressed proteins were diverse including structural, glycolysis related, signal transduction related, energy balance related, growth related, and stress related proteins. Some functional proteins important in optimal neuronal activity were also identified. Third, the affected proteins involved in signal transduction were mostly associated with Ca^{++} and/or Ca^{++}/calmodulin signaling pathway. Fourth, comparison of protein expression pattern between the groups exposed to single chemical and the groups exposed to chemical mixture suggested that pharmacodynamic interactions between PCB congeners and MeHg could alter the effects of each chemical.

Effects of PCB congeners and MeHg on the expression of functional proteins.

Even though developmental neurotoxicities by PCB congeners and/or MeHg have been known over 30 years, mechanistic bases for toxic actions are not clear. Many studies suggested that PCB congeners and/or MeHg may elicit neurotoxic effects by the induction of apoptosis and necrosis, fragmentation of microtubules and actin, disruption of neurotransmitter systems, induction of oxidative stress, disruption of calcium and thyroid hormone signal transduction, inhibition of cell proliferation and growth, and the delay of synaptogenesis (Tilson and Kodavanti, 1998; Porterfield,

2000). In our study, the expressions of many proteins were affected by perinatal exposure of PCB congeners and/or MeHg.

The characteristics of affected proteins were diverse, and most of them are known to be important in brain function. In particular, the most affected proteins were related to glucose or carbohydrate metabolism (i.e., aldolase 1, alpha-enolase, enolase 2, glyceraldehyde-3-phosphate dehydrogenase, and D-3-phosphoglycerate dehydrogenase). The importance of glucose in brain function has long been known. Glucose is the major energy source for the brain and is essential for maintaining normal brain function such as survival, learning, and memory (Messier and Gagonon, 1996). Some studies implied that certain neurological disorders were associated with the impairment of glycolysis (Hovda *et al.*, 1995).

The proteins involved in stress-related response were also affected by chemical treatements. Since stress response is the basic adaptive process for cell survival, it is thought that the affected proteins in this category may play roles in self-defense or adaptation against chemical challenge (Kopecek *et al.*, 2001). These proteins are antioxidant enzymes (glutathione peroxidase, thioredoxin peroxidase 1), proteolytic enzymes involved in ubiquitination (macropain, ADAM, ADAM 4 precursor, and toll-associated serine protease), the ones important in protein folding (peptidylprolyl isomerase A, and alpha glucosidase II), and molecular chaperon proteins (heat shock protein 40 kDa, eIF3, zinc finger protein, and hnRNP A2/ hnRNP B1).

Maintaining cellular structure and homeostasis is crucial for normal morphogenesis and survival (Deitmer, 2002). In the present study, the proteins involved in maintaining structural integrity (actin, internexin neuronal intermediate

filament, meltrin gamma, tubulin beta, profiling 2, and cofilin), energy balance (creatine kinase, and voltage-dependent anion channel 1) were affected by chemical treatments.

Three proteins involved in cellular growth and differentiation (follistatin-like, noggin, testis derived transcript) were affected by PCB congeners and/or MeHg. Follistatin-like proteins play a role in modulation of TGF superfamily signaling, which is important in cellular growth (Schneyer *et al.*, 2003). Testis derived transcript factor is important in the expression of sensory neurons (Sarafi-Reinach *et al.*, 2001).

Some of the proteins involved in the regulation of neuronal functions were affected by chemical treatments. RAB27A, neuron specific X11L, and synuclein are all related to the regulation of neurotransmission (Langford, 2002; Ho *et al.*, 2003; Wersinger *et al.*, 2003). Dihydropyrimidinase like proteins are important in axon guidance during neurodevelopment (Fukata *et al.*, 2002). These results suggest that neurofunctional deficits by PCBs or MeHg reported previously may be associated with the disruptions of these proteins.

Interruption of Ca^{++} and/or Ca^{++}/calmodulin signaling pathways by PCB congeners and MeHg. In addition to the diverse functional roles, one of the significant characteristics is that many proteins affected in our studies are involved directly or indirectly in Ca^{++} or Ca^{++}/calmodulin related signal transduction. Intracellular calcium plays an important role in brain function. In particular, it conveys signals involving the control of neurodevelopment, cellular functions, synaptic plasticity, and neuronal death (Brini and Carafoli, 2000). These affected

proteins can be categorized as follows; First, some proteins can regulate the levels of cytosolic Ca^{++}. These proteins are creatine kinase, peptidylprolyl isomerase A, ryanodine receptor type 2, and glycolytic enzymes (Xu *et al.*, 1995; Schiene-Fischer and Yu, 2001; de Groof *et al.*, 2002). Second, the expression and function of some proteins are regulated by the levels of cytosolic Ca^{++}. These proteins are cofilin, ubiquitin related proteases, and calmodulin (Davidson and Haslam, 1994; Mykles, 1998; Ikura *et al.*, 2002). Third, some proteins can play roles in the modulation of the proteins activated by cytosolic Ca^{++}. These proteins are voltage-dependent anion channel 1, and alpha-synuclein (Levy *et al.*, 2003; Steidl *et al.*, 2003).

Further western blot analyses also supported that Ca^{++} or Ca^{++}/CaM related proteins were affected by chemical treatments. In hippocampus, CaMK IV has been implicated in the regulation of CRE-dependent transcription. In CaMK IV deficient mice, activity-dependent CREB phosphorylation, c-fos expression, and other Ca++/CREB-dependent gene expression were significantly attenuated (Ho *et al.*, 2000; Kang *et al.*, 2001). Also, CaMK IV has been involved in long-term potentiation (LTP) in hippocampus (Kasahara *et al.*, 2001).

MAPK (=ERK ½) is activated by CaM-binding proteins as Ras-GRF and CaMK IV. In hippocampus, MAPK activation is critical for synaptic plasticity such as LTP and LTD induction in hippocampus (Wu *et al.*, 1999; Thiels *et al.*, 2002).
In hippocampus, phosphorylation of specific glutamate receptor (i.e., Glu R1, AMPA-R) by CaMK II enhances electric current, which is important in LTP induction (Soderling, 2000). In addition, CaMK II can phosphorylate SynGap, thereby potentiating the activation of MAPK pathway, which is also important for

synaptic plasticity (Soderling, 2000). Phosphorylation of CaMK II by cytosolic calcium plays an important role in the growth of neurites and the development of neuronal complexity (Borodinsky *et al.*, 2002).

In hippocampus, PKC enhances NMDAR activation via CAKβ/src tyrosine kinase cascade and may increase the number of receptors expressed in synapses, followed by the enhancement of synaptic plasticity. In addition, PKC provides a point of convergence of control of NMDARs and synaptic plasticity for a wide variety of G-protein coupled and growth factor receptors (MacDonald *et al.*, 2001).

Interactive effects of co-exposure to PCB congeners and MeHg. Our results suggested that there are interactive effects on protein expression between PCB congeners and MeHg. First, the net effects of PCB congeners and/or MeHg on protein expression were different between single chemical exposure and chemical mixture exposure. In general, co-exposure to PCB congeners and MeHg affected more proteins than single chemical exposure. Second, the expression pattern of each protein was different between single chemical exposure and chemical mixture exposure. For example, the expression of actin at PND 9 decreased in the group exposed to MeHg but increased in the group co-exposed to PCB 153 + PCB 126 + MeHg even though PCB 153 and PCB 126 did not have any effect on actin. This difference cannot be explained by the additive effects of the chemicals. The interactive effects may be explained from two different perspectives: First, there may be pharmacokinetic interactions between PCB congeners and MeHg for perinatal transfer. In this scenario, the concentration of a specific chemical in hippocampus region may be different between single chemical exposure and chemical mixture

exposure. Difference of internal dose in hippocampus may induce diverse effects on protein expression. Second, interactive effects on Ca^{++} regulation will induce expression changes. Bemis *et al* (2000) showed that co-exposure to PCBs and MeHg induced synergistic effects at low dose exposure and antagonistic effect at high dose exposure on the increase of intracellular calcium concentration. The functions and expressions of many proteins are dependent upon intracellular Ca^{++} levels (Brini and Carafoli, 2000). Thus, interactive effects on Ca^{++} regulation may induce the differences of protein expressions.

Time-dependent changes in affected proteins. Our results showed that some proteins were affected by chemical exposures in a time-dependent manner. We found three distinct characteristics: First, expression levels were affected by chemical treatments in a similar manner both at PND 9 and 30. For example, the expression of glyceraldehyde 3-phosphate dehydrogenase decreased in MeHg treated group and increased in PCB 153 treated and PCB 126 treated groups both at PND 9 and PND 30. Second, expression levels were induced first and inhibited later or vice versa. The expression of testis-derived transcript showed this pattern in PCB 153 treated group. It seems that this pattern may be caused by the compensation mechanism maintaining homeostasis (Radice *et al.*, 1998). Third, expression levels were affected only at PND 9 or PND 30. Most affected proteins showed this pattern. These results suggest that these proteins may have different sensitivities to chemical exposure at different time points during developmental period.

In summary, our results showed that co-exposure to MeHg and PCB congeners during the perinatal period elicited interactive effects on protein expression in a

specific brain region, hippocampus. Considering the potential importance in risk assessment, further studies will be needed to reveal the mechanisms of interactive effects and their relationships with the neurofunctional deficits.

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Table 6.1. Comparison of protein expression between single chemical exposure and chemical mixture exposure.

Time of assay	Chemical exposure	Number of proteins affected by chemical exposure	
		Increase	Decrease
PND 9	Single chemical	7	12
	Chemical mixture	7	14
PND 30	Single chemical	9	11
	Chemical mixture	8	12

Table 6.2. Interactive effects of co-exposure of MeHg and PCB congeners on the protein expression (At PND 9)

Protein expression	Chemical treatment		
	single	Chemical mixture	
	153	126	153+126
increase	Aldolase 1 Glyceraldehyde 3-phosphate dehydrogenase Neuron-specific X11L protein Profilin 2 Synyclein alpha Testis derived transcript Thioredoxin peroxidase 1	Aldolase 1 Glyceraldehydes 3-phosphate dehydrogenase hnRNP A2/hnRNP B1 Profilin 2 Testis derived transcript	Dihydropyrimidinase like 3 Thioredoxin peroxidase 1 Toll-associated serine protease Translation initiation factor eIF3
decrease	Macropain	Peptidylprolyl isomerase A	ADAM 4 protein precursor Phosphoglycerate dehydrogenase
	153	MeHg	153+MeHg
increase	Aldolase 1 Glyceraldehyde 3-phosphate dehydrogenase Neuron-specific X11L protein		Calmodulin 3 Cofilin Dihydropyrimidinase like 3

	Profilin 2 Synyclein alpha Testis derived transcript Thioredoxin peroxidase 1		Thioredoxin peroxidase 1 Toll-associated serine protease Translation initiation factor eIF3
decrease	Macropain	Actin Aldolase 1 Glyceraldehyde 3-phospate dehydrogenase hnRNP A2/hnRNP B1 Macropain Neuron-specific X11L protein Peptidylprolyl isomerase A Profilin 2 Protein kinase C binding protein Synuclein Testis derived transcript Thioredoxin peroxidase 1	ADAM Glutathione peroxidase Neuron-specific X11L protein Noggin Nuclear receptor subfamily 0 Testis derived transript
	153+126	MeHg	153+126+MeHg
increase	Dihydropyrimidinase like 3 Thioredoxin peroxidase 1 Toll-associated serine protease Translation initiation factor eIF3		Actin Cofilin Dihydropyrimidinase like 3 Thioredoxin peroxidase 1 Toll-associated serine protease
decrease	ADAM 4 protein precursor Phosphoglycerate dehydrogenase	Actin Aldolase 1 Glyceraldehyde 3-phospate dehydrogenase hnRNP A2/hnRNP B1 Macropain Neuron-specific X11L protein Peptidylprolyl isomerase A Profilin 2 Protein kinase C binding protein Synuclein Testis derived transcript Thioredoxin peroxidase 1	ADAM 4 protein precursor Aldolase 1 Dihydropyrimidinase like 2 Dihydropyrimidinase like 5 Phosphoglycerate dehydrogenase Glutathione peroxidase Glyceraldehyde 3-phosphate dehydrogenase Neuron-specific X11L protein Noggin Nuclear receptor subfamily 0 Profilin 2 Voltage-dependent anion channel 1

Table 6.3. Interactive effects of co-exposure of MeHg and PCB congeners on the protein expression (At PND 30)

Protein expression	Chemical treatment		
	single	Chemical mixture	
	153	126	153+126
increase	Translation initiation factor eIF3 Glyceraldehyde 3-phosphate dehydrogenase Heat shock protein 40 kDa RAB27A protein Thioredoxin peroxidase 1	Creatine kinase Follistatin-like protein Glyceraldehyde 3-phosphate dehydrogenase Macropain RAB27A protein	Alpha glucosidase II Actin beta Enolase 2 Ryanodine receptor type 2 Tubulin beta Zinc finger protein
decrease	Enolase alpha Creatine kinase Testis derived transcript	Enolase alpha Heat shock protein 40 kDa Testis derived transcript Thioredoxin peroxidase 1	Glyceraldehyde 3-phosphate dehydrogenase Heat shock protein 40 kDa Killer cell lectin like receptor Peptidylprolyl isomerase Regulator of G-protein signaling 17 Thioredoxin peroxidase 1 Voltage dependent anion channel 1
	153	MeHg	153+MeHg
increase	Translation initiation factor eIF3 Glyceraldehyde 3-phosphate dehydrogenase Heat shock protein 40 kDa RAB27A protein Thioredoxin peroxidase 1	Alpha glucosidase II	Meltrin gamma Ryanodine receptor type 2 Tubulin beta Zinc finger protein
decrease	Enolase alpha Creatine kinase Testis derived transcript	Enolase alpha Creatine kinase Translation initiation factor eIF3 Follistatin-like protein Heat shock protein 40 kDa Macropain RAB27A protein Testis derived transcript Thioredoxin peroxidase 1	Aldolase 1 Enolase alpha Peptidylprolyl isomerase Regulator of G-protein signaling 17 Testis derived transcript Voltage-dependent anion channel 1
	153+126	MeHg	153+126+MeHg
increase	Alpha glucosidase II	Alpha glucosidase II	Alpha glucosidase II

	Actin beta		Internexin neuronal intermediate filament
	Enolase 2		Ryanodine receptor type 2
	Ryanodine receptor type 2		Tubulin beta
	Tubulin beta		Zinc finger protein
	Zinc finger protein		
		Enolase alpha	
	Glyceraldehyde 3-phosphate dehydrogenase	Creatine kinase	
	Heat shock protein 40 kDa	Translation initiation factor eIF3	Enolase alpha
	Killer cell lectin like receptor	Follistatin-like protein	Calmodulin 3
decrease	Peptidylprolyl isomerase	Heat shock protein 40 kDa	Regulator of G-protein signaling 17
	Regulator of G-protein signaling 17	Macropain	Testis derived transcript
	Thioredoxin peroxidase 1	RAB27A protein	Voltage dependent anion channel 1
	Voltage-dependent anion channel 1	Testis derived transcript	
		Thioredoxin peroxidase 1	

Appendix 6.1. Identification of proteins significantly affected by single chemical exposure (At PND 9).

Spot No.	NCBI accession No.	Protein identification[a]	Amino acid sequence coverage (%)[b]	Possible roles[c]	Expression change (fold by control)[d]		
					PCB 153	PCB 126	MeHg
1	1363149	actin	31.5	cell motility	0.8±0.6	1.2±0.4	0.2±0.2
2	6671539	aldolase 1	38.2	glycolysis	2.6±1.3	2.2±1.4	0.1±0.2
3	6679937	glyceraldehyde-3-phosphate dehydrogenase	16.8	glycolysis	1.2±0.2	1.8±0.4	0
4	4504447	hnRNP A2 / hnRNP B1	10.8	mRNA processing	1.1±0.9	1.9±0.7	0
5	20843105	macropain	7.6	ATP/ubiquitin-dependent proteolysis	0.6±0.2	1.0±0.7	0.3±0.4
6	1351432	neuron-specific X11L protein	7.8	exocytosis of synaptic vesicle	1.8±0.1	0.9±0.9	0
7	20853425	peptidylprolyl isomerase A	32.3	acceleration of protein folding	1.0±0.1	0.7±0.2	0.4±0.4
8	9506971	profilin 2	20.7	regulation of actin polymerization	2.0±0.5	3.1±0.7	0.1±0.1
9	2494290	protein kinase C-binding protein	17.3	signal transduction	0.9±0.2	1.0±0.2	0.3±0.3
10	6678047	synuclein, alpha	15.4	regulation of dopamine release and transport	1.7±0.5	1.3±0.9	0.3±0.5
11	6755769	testis derived transcript	6.6	regulator of developmental pathways	2.5±0.9	1.9±0.3	0.2±0.3
12	2499469	Thioredoxin peroxidase 1	21.2	antioxidant	1.1±0.4	0.8±0.3	0.2±0.1

[a]Protein names are listed as the NCBI database.
[b]The portion of the protein containing matched peptide fragments from MS/MS analysis.

[c]Information were obtained from SWISS-PROT database.
[d]Values are represented as mean±S.D.

Appendix 6.2. Identification of proteins significantly affected by chemical mixture exposure (At PND 9).

Spot No.	NCBI accession No.	Protein identification[a]	Amino acid sequence coverage (%)[b]	Possible roles[c]	Expression change (fold by control)[d]		
					PCB 153 + PCB 126	PCB 153 + MeHg	PCB 153 + PCB 126 + MeHg
1	1363149	actin	31.5	cell motility	1.1±0.2	1.2±0.5	1.5±0.4
2	6752960	ADAM	10.1	non catalytic metalloprotease-like protein	1.2±0.5	0.8±0.2	1.1±0.3
3	2137121	ADAM 4 protein precursor	12.9	metalloprotease-like protein	0.5±0.3	1.0±0.3	0.5±0.4
4	6671539	aldolase 1	38.2	glycolysis	0.7±0.9	0.6±0.6	0.4±0.4
5	20820398	calmodulin 3	36.9	signal transduction	1.0±0.5	1.1±0.1	1.1±0.1
6	20884735	cofilin	27.1	regulation of actin polymerization	1.7±0.9	1.2±0.1	0.6±0.2
7	6753676	dihydropyrimidinase-like 2	22.2	axon guidance	0.7±0.5	1.0±0.1	0.8±0.1
8	6681219	dihydropyrimidinase-like 3	18.3	axon guidance	1.3±0.2	1.8±0.4	1.4±0.2
9	12746424	dihydropyrimidinase-like 5	19.0	axon guidance	1.1±0.3	1.0±0.8	0.4±0.2
10	28488414	D-3-phosphoglycerate dehydrogenase	11.8	glycolysis	0.6±0.2	0.9±0.2	0.7±0.3

11	121666	glutathione peroxidase	20.4	antioxidant	0.7±0.4	0.6±0.2	0.5±0.4
12	20819772	glyceraldehyde-3-phosphate dehydrogenase	30.6	glycolysis	0.9±0.2	1.1±0.2	0.8±0.1
13	1351432	neuron-specific X11L protein	7.8	exocytosis of synaptic vesicle	0.8±0.5	0.6±0.2	0.5±0.4
14	7110675	noggin	13.4	brain development	0.7±0.9	0.1±0.2	0.6±0.3
15	6671531	nuclear receptor subfamily 0	4.0	regulation of development	0.9±0.6	0.5±0.1	0.3±0.1
16	9506971	profilin 2	20.7	regulation of actin polymerization	1.0±0.4	0.8±0.5	0.7±0.3
17	6755769	testis derived transcript	6.6	regulator of developmental pathways	0.8±0.3	0.7±0.2	0.9±0.5
18	2499469	thioredoxin peroxidase 1	21.2	antioxidant	1.5±0.1	1.3±0.2	1.4±0.2
19	14994305	toll-associated serine protease	7.8	serine protease	2.5±0.8	1.9±0.9	2.0±0.6
20	9055214	translation initiation factor eIF3	11.2	promotion of translation	1.5±0.3	1.1±0.1	1.1±0.1
21	10720404	voltage-dependent anion channel 1	23.3	energy balance	0.9±1.4	1.1±0.3	0.2±0.2

[a]Protein names are listed as the NCBI database.
[b]The portion of the protein containing matched peptide fragments from MS/MS analysis.
[c]Information were obtained from SWISS-PROT database.
[d]Values are represented as mean±S.D.

Appendix 6.3. Identification of proteins significantly affected by single chemical exposure (At PND 30).

Spot No.	NCBI accession No.	Protein identification[a]	Amino acid sequence coverage (%)[b]	Possible roles[c]	Expression change (fold by control)[d]		
					PCB 153	PCB 126	MeHg
1	13637776	alpha enolase	3.9	glycolysis	0.7±0.1	0.6±0.1	0.3±0.1
2	6679465	alpha glucosidase II	5.6	regulation of folded proteins	0.9±0.2	1.2±0.5	1.8±0.1
3	10946574	creatine kinase	8.1	Energy transduction	0.7±0.2	1.2±0.1	0.4±0.1
4	9055214	eIF3	5.5	Initiation of translation	2.8±0.9	1.1±0.3	0.5±0.3
5	31560699	follistatin-like	6.9	Cell proliferation and differentiation	1.0±0.1	1.0±0.1	0.5±0.2
6	6679937	glyceraldehyde-3-phosphate dehydrogenase	12.0	glycolysis	1.5±0.1	1.7±0.7	0.2±0.2
7	17647497	hemoglobin	42.2	Oxygen transport	0.8±0.1	0.5±0.1	0.2±0.2
8	7949027	Hsp40	15.2	Molecular chaperon	1.0±0.1	0.8±0.1	0.1
9	20843105	macropain	21.3	ubiquitin-dependent proteolysis	0.9±0.1	1.1±0.01	0.4
10	13128964	RAB27A protein	15.4	vesicle-mediated transport	1.2±0.1	1.1±0.01	0.1±0.1
11	6755769	testis derived transcript	6.7	regulator of developmental pathways	0.6±0.2	0.6±0.2	0
12	6755758	thioredoxin peroxidase 1	17.2	antioxidant	1.2±0.2	0.9±0.1	0.4±0.2

[a]Protein names are listed as the NCBI database.
[b]The portion of the protein containing matched peptide fragments from MS/MS analysis.
[c]Information were obtained from SWISS-PROT database.
[d]Values are represented as mean±S.D.

Appendix 6.4. Identification of proteins significantly affected by chemical mixture treatment (At PND 30).

Spot No.	NCBI accession No.	Protein identification[a]	Amino acid sequence coverage (%)[b]	Possible roles[d]	Expression change (fold by control)[e]		
					PCB 153 + PCB 126	PCB 153 + MeHg	PCB 153 + PCB 126 + MeHg
1	6671539	aldolase 1	9.8	carbohydrate metabolism	0.7±0.6	0.4±0.1	1.0±0.7
2	13637776	alpha enolase	3.9	glycolysis	0.8±0.3	0.5±0.1	0.7±0.3
3	6679465	alpha glucosidase II	5.6	regulation of folded proteins	2.7±0.6	1.5±0.6	1.8±0.8
4	4501885	beta actin	31.5	cell motility	2.1±0.7	1.1±0.3	1.7±1.2
5	20820398	calmodulin 3	36.9	signal transduction	0.8±1.2	1.2±1.1	0.8±0.1
6	7305027	enolase 2	18.7	glycolysis	1.8±0.5	1.0±0.8	0.8±0.5
7	6679937	glyceraldehyde-3-phosphate dehydrogenase	12.0	glycolysis	0.6±0.2	1.0±0.4	1.0±0.8
8	7949027	Hsp40	15.2	Molecular chaperon	0.2±0.3	1.0±0.2	0.9±0.2
9	543250	Ig H chain	25.4	immunoglobulin	0	0.4±0.2	0.2±0.1
10	22122603	internexin neuronal intermediate filament	3.8	cell motility	1.5±1.1	2.3±1.8	2.2±1.2
11	17432435	killer cell lectin-like receptor	15.4	Immune function	1.2±0.8	0.6±0.2	1.4±1.5
12	2137514	meltrin gamma	17.2	cell-cell or cell-matrix interaction	1.1±1.8	2.8±1.3	1.3±1.9

13	20853425	peptidylprolyl isomerase A	32.3	acceleration of protein folding	0.2±0.1	0.5±0.2	1.2±0.8
14	2119534	ryanodine receptor type 2	4.7	Ca^{++} channel	2.0±0.8	1.3±0.1	1.5±0.3
15	9910532	regulator of G-protein signaling 17	21.4	signal transduction	0.4±0.2	0.8±0.1	0.7±0.3
16	6755769	testis derived transcript	6.7	regulator of developmental pathways	0.9±0.7	0.7±0.3	0.5±0.3
17	6755758	Thioredoxin peroxidase 1	17.2	antioxidant	0.2±0.4	0.9±0.2	0.8±0.7
18	21361322	tubulin, beta	41.4	Structural integrity	3.0±0.7	1.9±0.2	1.8±0.1
19	10720404	Voltage-dependent anion- channel 1	23.3	Energy balance	0.3±0.3	0.7±0.4	0.8±0.2
20	7106455	zinc finger protein	3.9	transcriptional regulation	2.7±0.3	2.1±1.0	2.0±0.8
21	20910025	Hypothetical protein[c]	17.0		0.1±0.1	0.7±0.2	0.7±0.1
22	20821527	Hypothetical protein	10.8		0.4±0.3	0.7±0.6	1.0±0.9

[a]Protein names are listed as the NCBI database.
[b]The portion of the protein containing matched peptide fragments from MS/MS analysis.
[c]Hypothetical protein is the protein sequence predicted from NCBI contig by automated computational analysis using gene prediction method.
[d]Information were obtained from SWISS-PROT database.
[e] Values are represented as mean±S.D.

CHAPTER 7

Discussion and Future Directions

Sun Ku Lee

I. Summary of the dissertation

The following significant finding and conclusions were drawn from this research.

1. Pharmacokinetic interactions between PCB congeners

Co-exposure to PCB 153 and PCB 126 increased the retention of PCB 153 in the liver and decreased the rate of PCB 153 accumulation in the fat of non-pregnant mice. However, co-exposure to PCB 126 and PCB 153 did not affect the extent of lactational transfer of PCB 153. In general, PCB 126 is assumed to exert its biological or toxicological actions through Ah-receptor mediated mechanism (Safe, 1984). The effects of co-exposure described above could be related to the Ah-receptor activation according to other studies (ATSDR, 1999b). Through the incorporation of effects of lipid accumulation in the liver and inhibition of lipoprotein lipase in the fat, the observed pharmacokinetic changes of PCB 153 in non-pregnant mice could be successfully simulated using PBPK modeling.

In contrast to the results for the non-pregnant mice, pharmacokinetic interactions between PCB 153 and PCB 126 were not evident in lactating mice and nursing pups. Pregnancy could induce many physiological changes in the mice. In

particular, the changes in the profile of plasma lipids and lipoproteins with late pregnancy could affect the disposition of PCB 153 significantly because PCB 153 is likely transported via lipoproteins with complex interactions (Spindler-Vomachka and Vodicnik, 1984). According to a previous study (Spindler-Vomachka and Vodicnik, 1984), the association profiles of PCB 153 with lipoproteins were changed during the perinatal period. Perhaps, these physiological changes can obscure the effects induced by PCB 126 as shown in non-pregnant mice.

2. Pharmacokinetic interactions between MeHg and PCBs

The present study demonstrated that co-exposure of the lactating mice to MeHg and PCB congeners could elicit pharmacokinetic interactions during lactational transfer from the mother to the pups. Through PBPK simulation, we concluded that experimental studies analyzing the relationships between the amount of chemical transfer and the level of transport protein in maternal blood will reveal the mechanisms of pharmacokinetic interactions between MeHg and PCB congeners. In the experimental study, the interactions were conspicuous for MeHg transfer. Overall, experimental results suggested that co-exposure with PCB congeners could increase the lactational transfer of MeHg to the pups. In addition, co-exposure with PCB congeners increased the lactational transfer of MeHg to the pups and compensated the plasma levels of albumin which decreased by the exposure of MeHg only. These results were matched with the simulation results, suggesting that MeHg could be transferred to the pups through binding with albumin in maternal blood and the different effects on albumin levels in maternal blood between the group exposed to

MeHg only and the group exposed to MeHg + PCBs may be one possible mechanism for pharamacokinetic interactions between MeHg and PCB congeners.

3. Pharmacodynamic interactions between MeHg and PCBs

A. In cerebellum

In our study, the expressions of many cerebellar proteins were affected by perinatal exposure to MeHg and/or PCB congeners. The characteristics of affected proteins were diverse, and most of them are important in brain function. The most affected proteins were related to glucose or carbohydrate metabolism. Glucose is the major energy source of brain and is essential for maintaining normal brain function such as survival, learning, and memory (Messier and Gagonon, 1996). The proteins involved in stress related response were also affected by chemical treatments. Since stress response is the basic adaptive process for cell survival, it is thought that the affected proteins in this category may play roles in self-defense or adaptation against chemical challenge (Kopecek *et al.,* 2001). In addition, many proteins are involved directly or indirectly in Ca^{++} or Ca^{++}/calmodulin related signal transduction. Intracellular calcium plays an important role in brain function. Western blot analyses supported the above results.

We suggested that there are interactive effects on protein expression between MeHg and PCB congeners. These suggestions were based on the following observations: First, the net effects of MeHg and/or PCB congeners on protein expression were different between single chemical exposure and chemical mixture exposure. Second, the expression patterns of affected proteins were different between single chemical exposure and chemical mixture exposure.

B. In hippocampus

We observed the following results through this study. First, perinatal exposure to MeHg and/or PCB congeners elicited differential expression of functional proteins in the hippocampus. Second, the differentially expressed proteins were diverse, including structural, glycolysis related, signal transduction related, enegy balance related, growth related, and stress related proteins. Some functional proteins important in optimal neuronal activity were also identified. Third, the affected proteins involved in signal transduction were mostly associated with Ca^{++} and/or Ca^{++}/calmodulin signaling pathway. Fourth, comparison of protein expression pattern between the groups exposed to single chemical and the groups exposed to chemical mixture suggested that pharmacodynamic interactions between MeHg and PCB congeners could change the effects of each chemical.

C. Comparison of the results between cerebellum and hippocampus

Even though there are many different subsections functionally distinct in brain, we focused on cerebellum and hippocampus for our pharmacodynamic studies since the chemicals we are interested in (i.e., MeHg and PCBs) have shown neurological and/or neurobehavioral deficits involving in sensory/motor functions and learning/memory function. In general, it is believed that cerebellum plays a role in coordinating sensory/motor function and hippocampus plays a role in learning/memory function (Gilbert, 2001). Thus, chemical effects on protein expression were observed in cerebellum and hippocampus.

We found that the perinatal exposure of MeHg and/or PCB congeners elicited expression changes of certain groups of proteins in both cerebellum and hippocampus.

These proteins are glycolysis-related proteins, Ca2+/calmodulin related proteins, stress related proteins, and cell structure related proteins. As we discussed in each sections, the interruptions of these proteins may be directly or indirectly related to the functional deficits by MeHg and/or PCB congeners.

Some of the proteins directly involved in neurotransmission were affected in hippocampus only. This result may suggest that specific functional deficits including learing and memory could be associated with these protein changes. Specific proteins were different between cerebellum and hippocampus even though these proteins are in same functional category. For example, phosphoglycerate mutase 1, a glycolytic protein, was affected in cerebellum, not in hippocampus. Another glycolytic protein, glyceraldehyde 3-phosphate dehydrogenase, was affected in hippocampus, not in cerebellum. These results may be caused by the different abundances of specific proteins between cerebellum and hippocampus.

II. Future directions

Electrophysiological and neurobehavioral studies. Through this research, we showed that perinatal exposure to MeHg and/or PCB congeners elicited the expression changes of functional proteins. In addition, we showed that the co-exposure of MeHg and PCB congeners induced interactive effects on protein expression. Even though we discussed the relationships between protein interruptions and functional deficits reported in previous studies, we did not show actual correlations between the effects on protein expressions and the effects on functional deficits. Thus, further studies investigating the relationships between the proteins interrupted and functional deficits induced by MeHg and/or PCB congeners will be

important in revealing the importances of specific proteins or relations between each group of proteins on brain function. For functional studies, we are considering electrophysiological assessments or behavioral assessments. Both MeHg and PCBs have been known to induce deficits in learning and motor activity (ATSDR, 1999a; 1999b). These effects could be caused by the damage in hippocampus and cerebellum (Annau and Cuomo, 1988). For the assessment of the functional damages in hippocampus and cerebellum, it is reasonable to carry out electrophysiological assessment. Sometimes, electrophysiological assessments are not matched well with functional deficits. Therefore, it is reasonable to perform behavioral assessments in addition to electrophysiological assessments. These studies will be important to ascertain whether the perinatal exposure of MeHg and PCB congeners could elicit neurofunctional deficits.

Pharmacokinetic studies for brain subsection. Brain is very complex organ both functionally and anatomically. Each sub-regions of brain has unique and different functions. For example, cerebellum plays a major role in motor control and cognitive functioning. In contrast, hippocampus plays an important role in learning and memory. Many research articles indicated that MeHg and PCBs induce functional deficits in psychomotor function, sensitivity, and learning/memory ability (Bemis and Seegal, 2000). These deficits can be related to the disruptions in cerebellum and hippocampus. Therefore it is important to monitor pharmacokinetics of the chemicals in brain subsections, especially cerebellum and hippocampus. This information will be useful in exploring dose- response assessment of MeHg and PCB congeners in developing animals.

Dose-dependent response on protein expression. Our studies on proteomics revealed that perinatal exposure to MeHg and/or PCB congeners affected the normal expression of proteins in the developing brain. Our next goal is to investigate dose-dependent protein expression changes. Even though the studies described herein provided useful information on protein expression changes, the studies were incomplete because we did not explore expression changes in a dose-dependent manner. Some studies suggested that protein expression patterns were different between animals exposed to low and high doses (Drake *et al.*, 2003). Therefore, it is important to investigate expression profiles of proteins affected by chemical treatments with diverse dose regimens. We will expose three different doses (i.e., low exposure, medium exposure, and high exposure) of MeHg or PCB congeners to the animals. In addition, animals will be administered to combinations of different dose of chemicals (i.e., low-low, low-medium, low-high, medium-low, medium-medium, medium-high, high-low, high-medium, high-high). As described in previous sections, proteins will be extracted and profiling will be performed by two dimensional electrophoreses and tandem mass spectrometry.

Comprehensive proteomics using updated technologies. For more extensive profiling and correct quantitation of proteins, state-of-the-art technologies, such as ICAT (isotope-coded affinity tagging), will be explored to compare the results from classical two-dimensional electrophoreses. To accomplish this task, two different samples will be treated with two different affinity tags, which are structurally identical except that one has a linker with eight hydrogen atoms, while the other has a linker with eight deuterium atoms. Thus one state of the cell is labelled with the light

tag, the other with the heavy tag (Kennedy, 2002). After labeling, the samples will be mixed and digested using trypsin. The only difference between these two peptides is that one is labeled with the light tag, the other with the heavy tag. Labeled peptides will be separated from bulk using affinity chromatography. Mass spectral analysis can be accomplished with electrospray ionization tandem mass spectrometry (LC-MS/MS). The relative ion intensities of the two differentially isotopically tagged forms of a specific peptide indicate their relative abundance (Han *et al.*, 2001). The protein from which a peptide originated is determined by searching a sequence database with its recorded tandem MS/MS spectra. Comparison of the results between ICAT methodologies and two dimensional electrophoreses will give complementary information on the changes of protein expression. If needed, more studies will be explored to validate which technologies give us more accurate and comprehensive information on protein expression.

Proteomics on post-translational modifications. Most proteins undergo post-translational modifications. To characterize these modifications is important since they may alter physical and chemical properties, foldings, conformations, and functions of proteins. Post-translational proteomics is an active area pursuing post-translations of proteins by high throughput techniques (Mann and Jensen, 2003). In our research, we initially focused on the expression changes of proteins following chemical treatment. However, it is also important to investigate post-translational modifications of proteins by chemical treatment. Both MeHg and PCB congeners have been known to interrupt cytosolic calcium signal transduction (Bemis and Seegal, 1999; 2000). Some of the effects may change the expression levels of certain

proteins. In addition, the interruptions of cytosolic calcium homeostasis will induce post-translational modifications of functional proteins, which may result in their activation or inhibition. In other words, MeHg and/or PCBs can modulate the function of proteins even though the chemicals do not change the expression levels of those proteins. Thus, we will explore the effects of MeHg and/or PCB congeners on post-translational modification of the proteins by known proteomics techniques. Initially, we will focus on phosphorylation, carboxylation, glycosylation, and sulfation. Then, we will extend to the other areas such as attachments of fatty acids, targeting, and cell-cell, cell-matrix interactions.

Biologically-based computational modeling linking complementary genomics/proteomics and biological function. Further research will be dedicated to the exploration of chemical effects on neurodevelopment through biologically-based computational modeling. Following the success of human genome project, many scientists have recognized the need to integrate the biological information obtained by high throughput technologies such as genomics and proteomics (Kennedy, 2002). In our studies, we explored the toxic effects using proteomics technologies. In addition, we described the need to perform more extensive proteomics studies. The next step will be the exploration of biological information using genomics technologies (e.g., c-DNA microarray). The results obtained by genomics will show us the complementary information as compared to proteomics. In particular, we expect to observe the so called "controversial results" which shows that the results by genomics do not always correlate well with those obtained by proteomics. These results will be valuable in understanding the

regulatory mechanisms of biological components affected by chemical exposure because the controversial results imply that regulation of the biological components will be different at the transcriptional, translational, and post-translational levels. Moreover, this information will give us insights regarding developmental regulation and its interruptions by developmental toxicants. All of this information will be interpreted more reasonably when they are integrated in a comprehensive way. Some of the research areas such as systems biology, Boolean network modeling, and reaction network modeling focus on the integration of biological information by computational/mathematical methodologies (Weiss *et al.,* 2003). In our research, these modeling areas will be explored in a sequential way.

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