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ABSTRACT OF DISSERTATION

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The Graduate School

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INTERACTIONS BETWEEN SELENIUM AND
POLYCHLORINATED BIPHENYLS (PCBs)

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in
The Graduate School
at the University of Kentucky

By
Divinia Nolasco Stemm

Lexington, Kentucky

Co-Directors: Dr. Howard Glauert, Professor
Dr. Larry W. Robertson, Professor, University of Iowa

Lexington, Kentucky

2005

ABSTRACT OF DISSERTATION

INTERACTIONS BETWEEN SELENIUM AND
POLYCHLORINATED BIPHENYLS (PCBs)

This study investigated the interaction between polychlorinated biphenyls (PCBs) and selenium to explain the mechanism involved that could affect selenium metabolism and its anti-cancer property. PCBs congeners and mixtures were previously found to reduce hepatic Se and Se-dependent glutathione peroxidase activity. I hypothesized that certain PCB congeners affect selenium metabolism in the rat liver resulting in diminished antioxidant capacity of selenoproteins, which could alter the ability of Se to protect against PCBs induced tumor promotion.

In the first study, the influence of 3,3',4,4'-tetrachlorobiphenyl (PCB 77) on hepatic Se and glutathione peroxidase (GPx1) activity as well as cytochrome P450 1A1 induction was examined by employing a time-course study, which showed that PCB 77 significantly reduced the hepatic selenium level and GPx1 activity and that this effect was influenced by gender. The next study explored how PCB 77 could deplete hepatic selenium by determining selenium concentrations in different tissues, feces and urine. This study demonstrated that PCB-77 decreased hepatic Se by increased excretion of Se in urine but not in feces. Unlike glutathione peroxidase, thioredoxin reductase activity was not affected by PCB 77. The third study investigated the effect of selenium supplementation on the tumor promoting activity of PCB 77 and 2,2',4,4',5,5'-hexaclorobiphenyl (PCB 153) using a 2-stage carcinogenesis model. Se supplementation did not diminish the induction of altered hepatic foci by coplanar PCB 77 or ortho-substituted PCB 153. Instead of protection, the number of foci per cubic centimeter and per liver among the PCB-77 treated rats was increased as the selenium dietary level increased. PCB 153 did not show the same selenium dose-response effect; nevertheless, selenium supplementation did not confer protection against foci development. On the other hand, supranutritional selenium reduced the mean focal volume. Supranutritional selenium or PCBs did not affect cell proliferation or thioredoxin reductase activity. Lastly, the use of the Zeeman graphite furnace atomic absorption spectrometry (GFAAS) method and closed microwave digestion technique for selenium determination of biological samples was compared with the neutron activation analysis and fluorometry methods. I found that GFAAS was not as reliable as the other methods.

KEYWORDS: Selenium, Polychlorinated Biphenyls, Glutathione Peroxidases,
　　　　　　　　Excretion, Tumor Promotion

DIVINIA NOLASCO STEMM

July 7, 2005

INTERACTIONS BETWEEN SELENIUM AND
POLYCHLORINATED BIPHENYLS (PCBs)

By

Divinia Nolasco Stemm

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DISSERTATION

Divinia Nolasco Stemm

The Graduate School

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POLYCHLORINATED BIPHENYLS (PCBs)

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CHAPTER 1. LITERATURE REVIEW ON SELENIUM

Properties, Bioavailability and Distribution

Selenium (Se) is an essential trace mineral that was discovered in 1817 and named after the Greek goddess of the moon, Selene, by Jons Jacob Berzelius, a Swedish chemist. Se is a metalloid found between bromine and arsenic in Period 4 and between sulphur and tellurium in Group VIA of the periodic table. Se is closely similar to sulfur in terms of chemical properties but with 3 differences. First, Se has various oxidation states, which enables it to be formed into organic compounds (methylated selenium) and into amino acids replacing sulfur (selenocysteine (Secys), selenomethionine (SeMet)). Second, Se forms a stronger acid than sulfur; the Se hydride or hydrogen selenide (H_2Se) has a pK_a of 3.7 while sulfur hydride has a pK_a of 6.9. Hence, the selenol form (R-SeH) can be easily dissociated at physiological pH making it important in catalytic reactions. Third, Se is metabolized into a reduced form while sulfur exists as an oxidized quadrivalent form. Se has been used in the manufacture of ceramics and glass, in xerography and in agriculture as a fungicide (seleno-diethyldithiocarbamate), in fertilizer to increase Se in plants for grazing animals, and in medicine as Se sulfide in shampoos used for treating tinea versicolor [1,2].

Se is an essential trace element. Schwarz and Foltz [3] discovered that a "Factor 3", which they found later to be Se, prevented liver necrosis in Se deficient animals. Disease conditions associated with Se deficiency were first recognized in farm animals. Se deficiency was found to produce liver necrosis in pigs, exudative diathesis in poultry, characterized by edema of body tissues; and nutritional muscular dystrophy or white muscle disease, a myopathy of heart and skeletal muscle in sheep and cattle. The effect of Se deficiency in humans was first recognized in the Keshan region of China where cardiomyopathy (Keshan disease) among young children and women of child bearing age was found to occur in areas where the soil is low in Se [4-6]. With supplementation of Se salt (as sodium selenite) incidence of Keshan disease declined. Another Se deficiency condition is osteoarthropathy or deforming arthritis condition, also called Kashin-Beck. Kashin-Beck is characterized by bone deformity produced by oxidative damage to the cartilage [7,8].

In animals, Se deficiency is associated with decreased glutathione peroxidase (GPx). GPx activity is affected by Se metabolism by increasing its synthesis and release in the liver [9-11]. At the same time plasma GSH is increased which can lead to depletion of cysteine and impaired protein synthesis. Uthus et al. [12] reported that Se deficiency decreased the plasma concentration of cysteine, cystathionine and homocysteine. Homocysteine can be used as marker for folate deficiency since Se deficiency can affect methionine metabolism [13,14]. Se deficiency could contribute to atherosclerotic cardiovascular disease by increasing thromboxane B2 leading to platelet aggregation and decreasing prostacyclin, which prevents platelet aggregation. Se deficiency was found to decrease some cytochrome P-450 isozymes while increasing uridine diphosphate glucuronyl transferase (UDP-GT) activity [15]. These effects could lead to increased toxicity or decreased efficiency of drugs.

On the other hand, too much Se can also be toxic. Se toxicity/intoxication falls into three clinical types: acute selenosis, subacute selenosis (i.e., blind staggers type), and chronic selenosis (i.e., alkali disease type) [16]. Acute poisoning occurs when high Se content plants are consumed in large quantities within a short period. Accidental acute poisoning in human occurs as a consequence of erroneous concentration of Se in a Se supplemented diet. Acute selenosis is characterized by garlic breath and lethargy, excessive salivation, vomiting, shortness of breath, muscle tremors and respiratory distress. Pathologies include congestion of the liver and kidney, fatty degeneration and focal necrosis of the liver, endocarditis and myocarditis. Sub-acute selenosis ("blind staggers") is caused by chronic poisoning by feeding on plants that are "natural Se accumulators" or primary indicator plants. These plants could contain as much as 1000-10,000 µg Se/kg mainly due to non-protein selenoamino acids, which these plants can synthesize from soil Se [17]. The disease is characterized by weight loss, neurological signs (blindness, ataxia, and disorientation) and respiratory disease. Chronic selenosis ("alkali disease") results from consumption of moderate levels of Se (5-40 mg/kg) for longer periods from plants categorized as "low Se accumulators" or secondary indicator plants. The clinical signs of chronic selenosis in horses, cattle and swine are hair loss (mane and tails of horses and cattle), emaciation, dystrophic changes in hooves, rough hair coat, and lameness. In advanced cases, liver cirrhosis, atrophy of the heart and

anemia occur. Unlike animals, plants can synthesize SeMet from inorganic Se in addition to other organic Se compounds [18].

For humans, Se toxicity reportedly produced hypochromic anemia and leucopenia and damaged nails in long-term exposed workers in a factory that manufactured Se rectifiers [19]. Accidental ingestion of selenic acid and vitamin tablets containing high Se was found to cause vomiting, diarrhea, hair and nail changes, and neurologic symptoms (acroparesthesis or pain, numbness and tingling in the extremities, weakness, convulsions, and decreased cognitive function) [19-21].

In 1973, Rotruck et al. [22] identified Se as a component of glutathione peroxidase, an enzyme previously found to be involved in hydroperoxide metabolism. Later on it was demonstrated that the Se in the glutathione peroxidase enzyme is a selenocysteine (Secys) residue that is integrated into the amino acid chain [23,24]. This Secys residue was shown to be essential for the catalytic mechanism [25,26].

The primary source of Se in soil is inorganic Se (mostly selenite). SeMet accounts for more than 50% of the total Se content in plants and the rest are Secys, methylselenocysteine and γ-glutamyl-Se-methylcysteine. Se found in foods exists in the form of SeMet, methylselenocysteine (MSC), Secys (mainly in animal sources) and selenate. Nutritional supplements consists mainly of selenate or selenite or Se-enriched yeast (SeMet and other Se compounds) [27-30]. Bioavailability and tissue distribution of Se depend on its form [1]. Since the total Se content of a particular source is not representative of its efficacy, the assessment of each selenium compound has been deemed necessary to determine which seleno-compound has beneficial effects on the health of humans and animals.

The Se contained in plants depend upon the soil in the region, that is whether it is seleniferous (selenium-rich soils) or Se-deficient soils [30-32]. When grown on seleniferous soils, vegetables like cabbages, potatoes, rutabagas and cucumbers can contain as much as 6 μg Se/g; however, some vegetables like onions and asparagus could accumulate up to 17μg Se/g. Se-enriched plants such as garlic, broccoli, onions and wild leeks predominantly contain methylselenocysteine (MSC). In enriched wheat, and in maize, rice, soybeans, and Se-enriched yeast, the predominant Se form is selenomethionine [30]. Meat products, breast milk, Brazil nuts and kidney are considered

to be good Se sources [33]. Crab, liver, some shellfish, and fish are moderately good sources of selenium. Fish intake does not necessarily increase Se status since different forms of Se exists in fish and Se interacts with mercury or arsenic, which are known contaminants in fish [34,35]. Se was shown to reduce the toxicity of some metals by forming inert metal selenide complexes. Mercury or methyl mercury was found to combine with Se and this binding is protective against mercury toxicity; however, this reduces the bioavailability of Se in foods mercury and Se [36-38].

The global distribution of Se is uneven and data is still lacking in many regions [39]. It is known that China has both the lowest as well as the highest Se areas in the world. Keshan in China is where Se deficiency in humans was first recorded [40]. In US, Se-deficient plants are found in the Pacific north-west, upper mid-west, the New England states and along the Atlantic coast. Seleniferous soils are found in the Dakotas, Colorado and western Nebraska [41]. Australia and New Zealand have reported Se-deficient regions [33,42-44]. Low-Se areas have also been reported in European countries; however, none has reported excess Se-containing areas. Other countries do not have available information. In Africa, a study found that AIDS prevalence appears to be higher in areas with low-Se condition [42-44].

Selenoproteins, selenoenzymes and Se-binding proteins

The term selenoproteins as used here refers to Secys-containing proteins and is not used for Se-binding or SeMet-containing proteins. Behne et al. [45] estimated that the selenoproteins and Se binding proteins could number from 30 to 50. At present, there are at least 25 selenoproteins that have been identified [46]. Only half of these 25 selenoproteins have been characterized in relation to biological function or enzymatic activity. The selenoproteins specifically incorporate the 21st amino acid, selenocysteine, and are responsible for carrying out most of the essential functions of Se.

Selenoprotein synthesis

Selenium in selenoproteins exists in the form of selenocysteine. All selenoproteins have one Secys except selenoprotein P which has 10 Secys residues. Secys is inserted into proteins in response to a UGA codon that commonly functions as a code for termination of protein synthesis [47-49]. The UGA codon has a Secys insertion sequence (SECIS) element, a RNA stem-loop structure, in the 3'-untranslated region of mRNAs in eukaryotes. Selenoproteins have equivalent sulfur-containing analogues or homologs where cysteine replaces Secys, but the SECIS element is missing in these homologs, which are less efficient and have a reduced range of substrates [50,51]. In the selenoprotein gene the in-frame UGA codons for Secys and SECIS element are located in 3'-UTR. In bacteria this is located downstream of UGA. In eukaryotes the mechanism for insertion has not been as well characterized as in bacteria although it is known that in eukaryotes, the SECIS elements bind 2 proteins, the SECIS-binding protein2 and an elongation factor [47]. The complex formed is responsible for recruiting Secys tRNA. In bacteria there is only one protein carrying out this function, the elongation factor SelB [47,48]. Secys tRNA is aminoacylated with serine and then using selenophosphate, a Se donor, it is converted to Secys by Secys synthase.

The eukaryotic selenoproteins are grouped on the basis of location and functional properties of the Secys. The function of the glutathione peroxidase enzymes as an intracellular defense against oxidative damage was the first widely recognized function of a selenoprotein. Later on, more functions for Se emerged, including its potential in cancer chemoprevention, reducing cardiovascular disease risk, preventing altered immune system functions and neurodegenerative diseases and preventing infertility, among others [52-54].

The selenoenzymes are Secys-containing proteins or selenoproteins, which could catalyze biological reactions. The selenoenzymes with known functions are: glutathione peroxidases (GPx), thioredoxin reductases (TrxR), iodothyronine deiodinases (DIO), selenophosphate-synthetase (Sps) and methionine-sulfoxide reductase B (MsrB) or selenoprotein R. Studies have also shown that Selenoprotein P (SePP) functions as a Se transport protein [55,56]. Selenoproteins with unknown functions include selenoprotein W, a 15-kDa selenoprotein and an 18 kDa selenoprotein.

Glutathione peroxidases (GPx1)

The first to be identified and most studied selenoprotein was the glutathione peroxidases (GPx) family. GPx catalyzes the reduction of hydrogen peroxide and lipid hydroperoxides to water and alcohols in the presence of reduced glutathione, which prevents production of reactive oxygen radicals and therefore protects from lipid peroxidation and cellular damage [22]. There are six isozymes that have been identified in human tissue: classical or cytosolic GPx (GPx1), gastrointestinal GPx2 (found in the liver and gastrointestinal tract), plasma glutathione peroxidase (GPx3), phospholipid-hydroperoxide GPx4 (PHGPx), sperm nuclei GPx5 (prostate), and GPx6 (in human only). The main role of the GPx1 as anti-oxidant has been confirmed by many studies [57-61]. The other GPx isozymes differ not only in tissue distribution but also in its specificity for peroxide degradation. In addition, GPx isozymes are involved in various metabolic processes including peroxynitrite scavenging [62] and arachidonic acid metabolism [63].

5'-Deiodinases (DIO)

Thyroxine (3,5,3',5'-tetraiodothyronine or T4) is the principal secretory product of the thyroid. The essential metabolic and developmental effects of T4 are all mediated by 3,5,3'-triiodothyronine (T3), which is produced from T4 by 5'-deiodination. The DIO-catalyzed 5'-deiodination of T4 to the biologically active T3 are mainly through the DIO Type 1 and 2 isozymes whereas the Type 3 isozyme is involved in the inactivation of the deiodination process [64-66]. The DIO isozymes differ in its function and tissue distribution. Type 1 is found mostly in the liver, kidney, thyroid and pituitary; Type 2 is mainly found in the brown adipose tissue, in placenta, pituitary and CNS; and Type 3 in skin, placenta and CNS [64,67]. The conversion of T4 to T3 is impaired in experimental Se deficiency which proves the essential role of Se in thyroid hormone action [68].

Thioredoxin reductases (TrxR)

Thioredoxin reductase (TrxR) is a member of the thioredoxin (Trx) system, a ≈12-kDa thiol/disulfide oxidoreductase. The thioredoxin enzyme family consists of the TrxR, the Trx, and the enzymes (peroxiredoxins, methionine sulfoxide) that derive their reducing equivalents from TrxR through Trx [46,69,70]. TrxR, a homodimer of ≈56-kDa

subunits, accepts a broad range of substrates, and like GPx differ by tissue distribution and intracellular location [71]. TrxR1 is found mainly in the cytosol (also known as cytosolic TR, TRα, and TrxR1), TrxR2 is localized in the mitochondria and TrxR3 expressed mainly in testis is also a mitochondrial TrxR [72-74]. The Trx system is involved in regulating the cellular redox status and redox regulation of transcription factors and hormonally-controlled nuclear receptors. In addition, reduced Trx is important in the ribonucleotide reductase catalyzed production of deoxynucleotides [73,75-77].

Selenoprotein W (Sep W)

A 10 kDa selenoprotein, Sep W, was first isolated from muscles [78]. Later study found it mainly in skeletal and heart muscles, brain, testis, and spleen and in smaller amounts in other tissues but not in liver, thyroid, pancreas, pituitary and eyes [79]. The function of Sep W is not known, but it is suggested that Sep W may have antioxidant function [80,81].

Selenophosphate-synthetase 2 (Sps2)

In *Escherichia coli,* Se is inserted into Se-dependent enzymes and Se-containing tRNAs through the activity of the *selD* gene product [82]. This product, later identified as selenophosphate synthetase (Sps2), forms a highly reactive, reduced Se compound, selenophosphate. Sps2 catalyzes the reaction in which a gamma phosphoryl group is transferred from ATP to selenide forming a reactive, reduced Se compound, selenophosphate, and AMP [83]. In eukaryotes, a pair of *Sps* genes exists; one encodes a selenoenzyme capable of *in vitro* catalysis, and the other homolog encodes an enzyme exhibiting poor catalytic activity for selenide-dependent hydrolysis of ATP. It was observed that Sps mRNA levels were elevated in organs previously implicated in selenoproteins synthesis [84]. Tamura et al. [85] hypothesized that *Sps2* may be involved in *de novo* synthesis of selenophosphate from selenite after reduction of selenite by intracellular thiols. The reduced Se binds to the Sec residue of *Sps2* forming an enzyme-substrate complex, leading to an increase in the cellular Se pool [85]. Selenophosphate is the Se donor for the biosynthesis of Secys [86].

Methionine-sulfoxide reductase B (MsrB) or selenoprotein R

Mammals contain two methionine sulfoxide reductases, MsrA and MsrB, that catalyze the thioredoxin-dependent reduction of R-methionine sulfoxide to methionine [87]. MsrB is a selenoproteins, indicating another potential redox-regulatory role of Se in mammals [88,89]. The MsrA enzyme contains a cysteine instead of a Secys residue. Mice grown on a Se-deficient diet demonstrated a decrease in the levels of MsrB-catalytic activity, MsrB protein, and MsrB mRNA in liver and kidney tissues of both wild type and MsrA-/- mouse strains [88,89]. A link between methionine oxidation and methionine-sulfoxide reduction and the aging process as well as reversible modifications for signaling proteins has been suggested [90].

Selenoprotein P (SePP)

SePP is an extracellular glycoprotein that contains Secys residues ranging from 10 residues in mice and humans to 17 residues in zebrafish compared with majority of selenoproteins which has a single Secys [91-93]. Predominantly a plasma protein, SePP binds 60-70% of the Se in plasma [94]. It is estimated that plasma SePP accounts for 8% of the total Se in the animal. The liver is the primary source of plasma SePP although other tissues could also express it [95]. Plasma SePP accounts for 8% of the total Se in the animal. Since plasma Se half-life is only 3-4 hours, Se cycles through plasma SePP at a high rate [96]. There is no direct evidence that the Secys residue of SePP has a chemically active role; however, several in vitro and in vivo studies have indicated the role of SePP as a Se transport or supply protein [55,96-100].

Knock-out models for SePP have shown that this plasma selenoprotein is essential in Se tissue distribution [94]. Moreover, SePP synthesis and secretion affected the Se levels in blood and tissues as well as the expression of GPx and TrxR [55,101-103]. The transport mechanism in plasma was demonstrated to be due to hepatically-derived SePP; however, the transport of Se in the brain may be due to a SePP-independent uptake route for Se [102]. Saito et al. [104] showed that SePP is a bi-functional protein and proposed that the first Secys residue is the enzyme active site while the remaining residues act as Se suppliers. SePP also serve as source of Se for Jurkat cells and embryonic neurons

[98,100]. Additional roles for SePP have also been proposed including antioxidant function [105-107] and extracellular storage depot [56,105,108].

Sep 15

Kumaraswamy et al. [109] recently found a 15 kDa selenoprotein (Sep 15), which was located at chromosome 1p31, a genetic locus that is altered in some human tumors. This selenoprotein was found together with a protein that is involved in regulating protein folding [110]. Sep 15 was expressed highly in the prostate, liver, kidney, testis and brain in mice. The protein level of Sep 15 was observed to be greatly reduced in malignant prostate cell lines as well as hepatocarcinoma [109]. It was suggested that Sep 15 may have a role in cancer development or risk.

Selenomethionine (SeMet) and Se binding proteins (SeBP)

Se in the form of selenomethionine is incorporated non-specifically in body proteins in place of methionine. During protein synthesis, SeMet is loaded directly onto methionine transfer RNA (tRNAMet) and replaces the essential amino acid Met in the growing peptide chain. The SeMet content of newly synthesized proteins is directly related to the ratio of SeMet to Met that is available for the methionyl-tRNA synthetase [111]. It has been proposed that since SeMet can be incorporated into the body proteins in place of Met, it is possible that SeMet could function as a reversible Se storage in organs and tissues. In contrast, it has been suggested that excess SeMet could be transformed directly into methylated Se by the gamma-lyase enzyme [112].

In addition to selenoproteins, Se can bind to certain proteins to form the so-called Se binding or containing proteins that themselves do not have Se as a structural component [46,113,114]. These are: an 8-kDa selenium-containing protein found in mitochondrial membranes, SelT (18.8 kDa), SelN (58 kDa), SelX (16 kDa), and 3, 4, 5, 7 kDa selenocysteine-containing proteins among others. Currently these proteins have no known function.

Se Uptake, Storage and Metabolism

The total body Se content in man is approximately 0.15 mg/kg. The adequate Se intake is estimated to be 50 µg/day. The toxic level for Se is estimated to range from 350-700 µg/day. The U.S. Environmental Protection Agency (EPA) limit for Se, assuming a life-time of exposure, is 5 µg/kg body weight/day. The low adverse effect level (LOAEL) for Se has been calculated at 1,540±653µg/day while the no adverse effect level (NOAEL) has been calculated at 819±126µg/day [115]. Chronic feeding of more than 5.0 mg Se/kg diet or parts per million (ppm) was found to cause teratogenicity and hepatoxicity in animals and humans. The recommended dietary allowance (RDA) for selenium is set at 55 micrograms per day for adults by the Institute of Medicine with an upper limit of 400 micrograms per day; although selenium supplementation is not deemed necessary in the United States since estimates of selenium intake are 80 to 120 micrograms, which is greater than the RDA [116].

General Metabolism Pathway

Se is normally taken up from the gut mainly as SeMet, Secys, selenate, and selenite. The bioavailability of the absorbed Se does not only depend on its form but also on its conversion into a biologically active form that can be utilized by tissues, retained in the tissue and conserved by the renal system [13]. With adequate intake, most of the absorbed Se is taken up by the liver and used for synthesis of hepatic selenoproteins [94,100].

The absorbed seleno-compounds are converted to hydrogen selenide (H_2Se), which is either converted to selenophosphate (which in turn reacts with a tRNA loaded with serine to tRNASecys) or methylated. Different intermediary metabolites are synthesized during the process of converting inorganic Se to organic forms and vice versa. Hydrogen selenide (H_2Se) is the key metabolite formed after inorganic sodium selenite (oxidation state +4) is processed into selenodiglutathione (GSSeSG) via reduction by thiols and NADPH-dependent reductases. Secys formed from SeMet is converted to H_2Se by β-lyase [117-119]. All Se compounds that are catabolized into hydrogen selenide eventually undergo methylation and are secreted in breath as dimethylselenide (DMSe) or in urine as monomethylated Se (MSe) or

trimethylselenonium ion (TMSe) [118]. A modified metabolic pathway for Se is shown in Figure 1.1 [1,118,120].

Using the neutron activation analysis (NAA), the selenium content of rats fed with a selenium-deficient diet (2-5 µg Se/kg) or Se-sufficient diet (300 µg/kg) were determined and compared with animals administered in vivo with radioactive ^{75}Se tracer [121]. The Se content of muscles, liver, erythrocytes and plasma of the Se-sufficient diet fed rats were found to be 620, 2660, 1540, and 4760 µg/kg dry sample respectively while the same tissues in the Se-deficient rats showed <9, 17, <10, and 34 µg Se/kg dry sample respectively.

Metabolism of inorganic Se (selenite and selenite)

Inorganic Se (which is usually not a major source of dietary Se) is more readily available to selenoprotein synthesis than SeMet, the major form of dietary Se from many plants. Se from selenate and selenite (used as a Se supplement in most animal studies) is more available metabolically since these could be easily reduced to selenide, the intermediate Se metabolite needed to synthesize Secys, the active form of Se in selenoproteins. Chemoprevention studies in animals have mostly used sodium selenite or selenomethionine as test agents. Suzuki and Ogra [122] determined the concentrations of ^{82}Se in organs and body fluids after intravenous administration of ^{82}Se-selenite and selenate to rats. They observed that selenite uptake by red blood cells occurred within several minutes, followed by reduction to selenide by glutathione, binding to albumin in the plasma, and then transport to the liver. On the other hand, selenate was either taken up directly by the liver or excreted into the urine. They proposed that the difference in metabolic pathways may be a result of regulation through selenide, the common intermediate metabolite for the inorganic and organic Se, which serves as the precursor metabolite for selenoprotein synthesis and for methylation into compounds that are excreted in urine.

Figure 1.1: Selenium Metabolism

selenite, selenate

Se^{4+}, Se^{6+}

Selenite

TrxR   GSH

GSSeGS

GSH or TrxR

Selenide

Selenophosphate

CH_3Se^-
methylselenol

GSH
or TrxR

CH_3SeO^-
methylselenenate

$(CH_3)_2Se$

$(CH_3)_3Se^+$

selenocysteine (Secys)
selenomethionine (SeMet)

SeCys   **SeMet**

SeCys   **Methionine pool**

$tRNA^{Sec}$

Selenoproteins

$tRNA^{Sec}$
loaded with serine

GSH
or TrxR

CH_3SeO_2
Methylseleninate
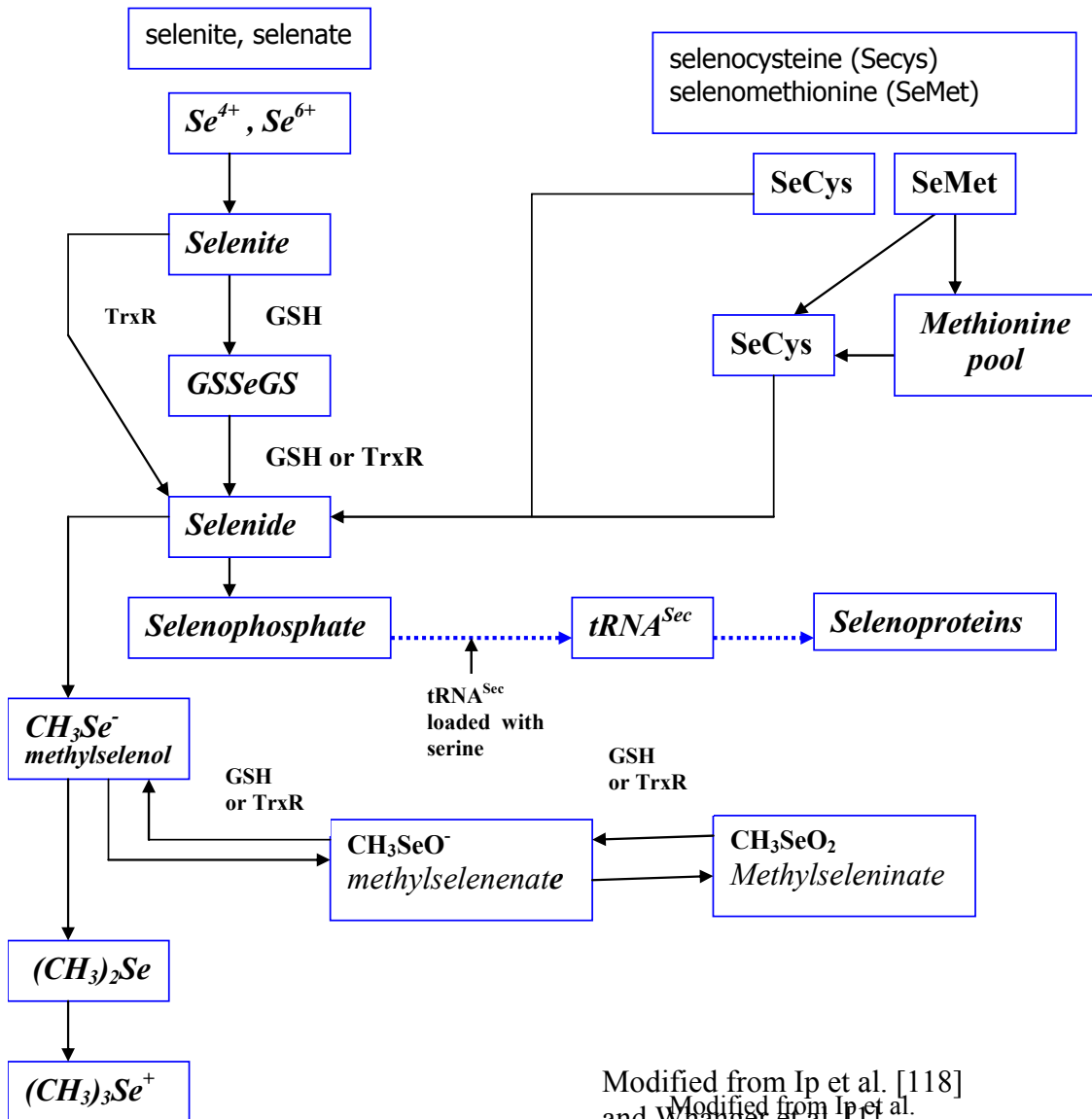
Modified from Ip et al. [118]
and Whanger et al. [1]

Metabolism of selenomethionine (SeMet)

Dietary SeMet is the most available form of Se since it can effectively increase apparent Se status owing to its non-specific incorporation in place of Met into proteins (e.g., haemoglobin, albumin). In mammals, SeMet appears to be absorbed in the small intestine through the Na^+-dependent neutral amino acid transport system [123]. Supplementation studies using Se-yeast have shown that Se was more bioavailable even after supplementation because SeMet is non-specifically incorporated into tissue proteins (skeletal muscles, albumin, erythrocytes), where it could later be catabolized and released to maintain increased Se status [1]. SeMet and selenite have an estimated half-lives of 252 and 102 days, respectively, in human subjects. This indicate that SeMet is retained longer in the body than selenite [124]. However, it has been argued that SeMet is less metabolically available because it requires catabolism to an inorganic precursor before it could enter the available Se pool [113,125]. The organic forms such as Se yeast are preferred in intervention studies because they are less acutely toxic, although due to its non-specific retention in body proteins, long-term consumption could produce toxicity [1,30,52,126-128]. Unlike SeMet, other organic Se compounds including Secys, methylselenocysteine (MSC) and γ-glutamyl-Se-methylcysteine are not incorporated non-specifically into proteins.

SeMet is catabolized in 2 possible pathways. The transsulfuration pathway produces Secys via selenocystathionine, which is then degraded to H_2Se by the β-lyase enzyme for subsequent methylation by methyltransferase. The other pathway is the transamination decarboxylase pathway, the main pathway for Met metabolism, which is therefore thought to be the major pathway for SeMet catabolism [129].

Metabolism of organic Se (Secys, MSC)

Se from animal sources is mostly present as Secys in a form which is not a direct precursor for selenoprotein synthesis. Se is liberated from Secys via Secys-β-lyase [130]. It is believed that to prevent high levels of Secys, which would lead to replacement of cysteine by Secys, Secys is converted to methylselenocysteine (MSC), which is then cleaved to methylselenol (CH_3SeH) [13,131].

MSC occurs naturally in plants including Se accumulating Astragalus species, garlic, broccoli, leeks, and onions. MSC from Se-enriched garlic is cleaved by β-lyase to form methylselenol (CH$_3$SeH) [132]. Compared to SeMet, MSC is not non-specifically incorporated into proteins, which is thought to be the reason why it appears to be more effective in cancer chemoprevention compared with other seleno-compounds [133,134]. MSC toxicity is comparable to that of SeMet [119]. The presence of β-lyase in liver, kidney, and intestine facilitates the metabolism of MSC to CH$_3$SeH [135]. In addition, compared to SeMet, MSC is readily available for further metabolism since it is not incorporated into proteins. On the other hand, MSC accumulation in tissues is lower and elimination is more rapid once MSC supplementation is stopped.

Methylation and excretion

Methylation is a major pathway for Se metabolism in microbes, plants and animals. Excess Se and degraded selenoproteins are transformed to methylated compounds, such as monomethylselenol (MSe) also believed to be the active metabolite methylselenol, dimethylselenide (DMSe) and trimethylselenonium ion (TMSe), for excretion in urine [136,137]. In animals, demethylation back to inorganic Se has been observed [138,139]. Se is excreted mainly via the urinary tract as monomethylselenol and trimethylselenonium ion. At higher concentrations, Se is exhaled as dimethylselenide, responsible for the garlic-like odor in Se intoxication.

After administering ^{75}Se-labeled selenite by gastric intubation to fasting rats, it was shown that trimethylselenonium ion (TMSe) production was dose dependent and that TMSe produced from a single acute dose was not affected by prior Se status [140]. Urinary profiles of rats with various Se status was investigated after i.v. injection of [82]Se-selenite [141]. The results demonstrated that metabolism of Se was dose-dependent. Moreover the primary metabolite of Se in untreated animals was MSe while TMSe was detected after injection of [82]Se-selenite. Vadhanavikit et al. [139] administered sodium selenite and five methylated Se compounds orally to female rats and observed that MSe was the main metabolite of selenite at low dose (0.1 ppm in the diet), although increasing the dose to 3 ppm Se increased the excretion of DMSe and TMSe sharply. When similar chemopreventive doses of mono-, di-, or trimethylated compounds (MSC, selenobetaine

methyl ester and selenobetaine, respectively) were administered, the methylated metabolites were observed to be more than that of selenite. In this study, excretion of inorganic and MSe indicated that demethylation occurred.

In male F344 rats treated p.o. with 4.35 mg Se/kg (as Na_2SeO_3), the cumulative percentages of doses excreted within 3 days of Na_2SeO_3 treatment were $18.33 \pm 0.77\%$ in urine; and $31.14 \pm 4.66\%$ in feces [142]. TMSe urinary metabolite was reported to be excreted in a dose dependent manner when the dose is 1.5 mg Se/kg body weight or lower, and then the level is maintained at higher doses [140]. In 12-hour urine of male Wistar rats injected with sodium selenite (400 μg Se/ml saline) at different doses (0, 0.1, 0.3, 0.5 and 1.0 mg/kg body weight), selenite metabolites namely MSe, TMSe, selenate and selenite were analyzed with HPLC using an inductively-coupled plasma-mass spectrometer (ICP-MS) as the detector [136]. In contrast, this study found that the excretion of TMSe did not level off at a Se dose of 1.0 mg Se/kg body weight. The total Se in urine in mice orally administered with different doses of selenocystine was determined using the fluorometric method [143]. In this study, the total Se in the 24-hour urine of animals with normal Se methylation process was lower than those with reduced methylation ability. In addition, a negative correlation was found between the concentration of the methyl donor, S-adenosyl methionine [144], and TMSe in liver.

Se and Cancer

Se has been shown to be effective in reducing cancer incidence in animal models and epidemiologic data as well as supplementation trials support the hypothesis that Se is likely to be effective in humans. Epidemiological studies on the relationship between Se and cancer have found that Se status is inversely related to some cancer risks. Shamberger reported this association in human subjects and also found that mortality attributed to lymphomas and cancers of the gastrointestinal tract (GIT), peritoneum, lung and breast were lower in subjects living in areas where Se concentration is high in forage crops compared to those living in areas with low-Se containing forage crops [145,146]. Clark and Stafford [147] using the same forage data, indicated that colorectal cancer mortality is indeed associated with high Se. Using the estimated Se intake per capita, Schrauzer et al. [148] noted an inverse association with total cancer mortality rate and

age corrected mortality rate for leukemia and cancers of the colon, rectum, breast, ovary and lung.

Using serum Se level, several studies reported that low serum or plasma Se level is associated with increased risk for some cancers such as GIT cancer, prostatic cancer, thyroid cancer, malignant oral cavity lesions, esophageal and gastric cancers, cervical cancer and colo-rectal adenomas, and non-melanoma skin cancer [149-156]. On the other hand, some studies have reported no significant association between serum Se concentration and cancer risks [157-160]. Trials using topical application of L-selenomethionine alone or combined with vitamin E demonstrated protection against acute u.v. irradiation effects on skin and skin cancer in human subjects [161-163].

Se supplementation trials have been conducted to determine if Se is effective in reducing certain cancers in human. Five of the eight supplementation trials were based in China and the rest in the USA, Italy and India. The first China trial investigated the preventive effect of Se on primary liver cancer and found that Se supplementation using table salt fortified with sodium selenite (30-50 μg Se/day) resulted in an almost 50% decrease in the primary liver cancer incidence [164,165]. Another study showed that selenite fortified salt supplementation reduced the incidence rate of viral infectious hepatitis, a predisposing factor of primary liver cancer [166,167]. Yu [165] reported a significant decrease in primary liver cancer among those receiving Se yeast (mainly selenomethionine) compared to controls. In contrast, two studies found that the treatment combination of Se as Se-enriched yeast, Vitamin E and β-carotene provided modest protection against esophageal and stomach cancer mortality [155,168]. It is thought that Se did not produce significant effects because the 50 μg Se/day dose may not be adequate dose to provide cancer protection.

A double-blind, randomized trial of Se-enriched yeast involving 1,312 patients with non-melanoma skin cancer led to the unexpected discovery that Se protects against colon, lung and prostate cancers [20,169]. After extending the trial to ten years, the resulting trend was still the same [170]. They also found that Se decreased the incidence of total cancer and prostate cancer, but not lung and colo-rectal cancers. However, contrary to results from epidemiological studies, Duffield-Lillico et al. [170] found that subjects with low plasma levels had lower incidence of cancer whereas those with high

plasma Se levels did not correlate with cancer incidence. To explain this discrepancy, they proposed that the plasma levels may have a threshold above which the effect is not protective against cancer. The results from this trial encouraged clinical intervention trials including Se and Vitamin E Cancer Prevention Trial (SELECT) in US and Prevention of Cancers by Intervention with Se (PRECISE) in Europe [171,172].

Se supplementation studies in animals have shown a beneficial effect of high Se against cancer [173,174]. Almost two-thirds of animal studies have demonstrated that Se significantly reduces tumor incidence [173]. Chemopreventive effects of Se have been observed for cancers of the mammary gland, liver, skin, pancreas, esophagus and colon [118]. Animal studies involving tumor initiation, promotion and progression in general support the anti-cancer effect of Se although individual Se compounds have differing protective effects [134,139,175]. It is believed that Se may be more effective in preventing rather than treating or reversing tumors as shown by animal studies [118]. It is estimated that the anti-tumor effect of Se of various forms (selenite, SeMet, Sec, SMC, selenobetaine and selenobetaine methyl ester) could inhibit tumor production by 50% if given at Se doses ranging from 2-5 ppm [30]. It is estimated that at a Se intake of 100 μg Se/kg body weight, anti-tumor effect of Se occurs in rodents.

In the mammary and liver tissues, the tumor initiator 7,12-dimethylbenz(a)anthracene (DMBA) is metabolized to reactive diol epoxides, which are involved in DNA adduct formation. Selenite inhibited the DMBA-induced mammary tumors and reduced the MBA-DNA adducts in rats [176-178]. DMBA-DNA adducts were also inhibited by dietary 1,4-phenylenebis(methylene) selenocyanate (p-XSC) [179]. On the other hand, selenite supplementation was found to have no effect on the DMBA- DNA binding in the mammary tissue or liver in rats[180]. In two separate studies, selenite was found to inhibit DNA adduct formation in rat liver after treatment with 2-acetylaminofluorene (2-AAF) or aflatoxin B1 (AFB1) [181-183]. In chicken liver, selenite did not inhibit the production of DNA adducts by AFB, which indicates that the species of animal affect the protective effect of Se [181].

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Se and Chemopreventive Mechanisms

Undoubtedly, Se can be anti-carcinogenic, but the mechanisms by which Se prevents cancer remain unclear. Several hypotheses have been proposed, including the chemoprotective effects of certain selenoproteins (GPx, SePP, TrxR) and Se metabolites (GSSeSG, MMSe). Mechanisms that have been put forth include inhibition of tumor cell proliferation, increased apoptosis, inhibition of tumor angiogenesis, alteration of carcinogen metabolism, and enhanced immune response [1,118,134,173,184]. Different supplementation forms of Se including inorganic Se salts, SeMet, Sec, SMC and synthetic Se compounds have been studied to find the most effective metabolically active form of Se that would be chemopreventive and yet non-toxic.

Combs et al. [185] summed up the anti-cancer mechanisms of cancer in two possible ways. One is through the anti-oxidant and redox regulating function of the selenoproteins particularly the selenoenzymes, which is suggested to be more protective against cancer initiation; and second, the production of anti-tumor selenium metabolites, which is more active against cancer progression. Thus, although other mechanisms may account for the anti-cancer effects of Se, the antioxidant properties of selenoproteins are also responsible for chemoprotection. The two major selenoenzymes, GPx and TrxR, are essential antioxidant components of 2 major redox systems in the mammalian cell: the glutathione and thioredoxin systems. In several studies, the observed chemoprevention was not associated with protection resulting from maximum production or activity of glutathione peroxidases or thioredoxin reductases indicating that the anti-tumorigenic effect of Se may not be associated with protection from selenoproteins [186-189]. Although GPx prevents oxidative stress, GPx activities reach a plateau at Se intake levels (0.1–0.4 ppm) that is not chemopreventive for cancer, which is 2–4 ppm [118,190-194]. Nevertheless, we can not dismiss the fact that the antioxidant properties of selenoproteins have a role in reducing oxidative damage and carcinogen generated reactive oxygen species (ROS) [195-198]. GPx catalyzes the reduction of hydrogen peroxides and lipid hydroperoxides at the expense of 2 molecules of reduced glutathione (GSH) in the presence of NADPH [199]. The oxidized glutathione, GSSG, is then regenerated by glutathione reductase. A study on use of selenite to protect against u.v. light or phorbol esters-induced skin tumors found that GPx activity in the skin correlated with tumor

protection [200,201]. GPx knock out mice did not produce abnormalities when subjected to hyperoxic conditions, hence, it is believed that a compensatory mechanism may exist for the loss of GPx1 function [202]. It has also been shown that overexpression of GPx1 in human T47D carcinoma cells inhibit the TNF-α and H_2O_2 mediated activation of NF-κB [203]. Gladyshev et al. [204] found that GPx1 expression is decreased in tumors compared to the surrounding normal tissue. The effect on GPx1 is explained by the increased production of ROS in the pre-neoplastic stage. Evidence that Gpx1 is not involved in chemoprevention was shown when *Gpx1*-null mice did not show any abnormal histopathologies yet they were sensitive to exogenous oxidative stress [202,205,206]. *Gpx1*-overexpressing transgenic mice were also found to be more sensitive to dimethylbenz[*a*]anthracene-12-*O*-tetradecanoylphorbol 12-acetate-promoted skin cancer [207]. They hypothesized that this may be caused by induction of tumor-promoting lipoxygenase-derived peroxides.

Thioredoxin reductase (TrxR) reduces thioredoxin (Trx) and the activated Trx controls cellular redox processes such as transcription, protein-DNA interactions, growth control and DNA synthesis [208,209]. Some studies demonstrate the protective role of the Trx system in cancer; however, some raised indications that this system may also have pro-tumorigenic effects [73,189,204]. Trx has been shown to inhibit apoptosis which therefore favors tumor growth [210]. TrxR activity in rat liver was not affected by high MSC or methylseleninic acid intakes. TrxR1 mRNA is repressed in cells overexpressing p53 [204]. Yet an active TrxR is necessary for normal and correct maturation of p53 which points to a possible role of TrxR in cancer protection via p53 [211]. Gladyshev et al. [204] studied the expression of GPx and TrxR in TGFα/*c*-myc mice and observed that TrxR expression and activity were increased in tumors [212]. In human prostate cell lines, TrxR was increased in cancer cells compared to the normal cells.

Another mechanism by which Se compounds could prevent tumor cell growth is apoptosis. Lanfear et al. [213] showed that cells treated with the initial selenite metabolite selenodiglutathione, a natural metabolite of selenite, induced p53 accumulation in cells containing wild-type p53. Later studies showed that Se compounds induced growth arrest or apoptosis of cells that express mutant p53 or lacking p53 completely [214]. This

contradicts the mechanism in which a functional wild-type p53 pathway is needed by Se compounds to inhibit cell growth and induce apoptosis. Instead Kaeck [214] suggested that the induction of *gadd34*, *gadd45*, and *gadd153 by* inorganic and organic forms of Se interfered with the cell cycle check point controls that are associated with growth arrest/apoptosis. Harrison et al. [215] found that a natural metabolite of selenite, selenodiglutathione, did not induce oxidative stress. They proposed that growth inhibition or apoptosis by selenodiglutathione may be due to a Se-resistant variant or a novel Se-binding protein. Powis et al. [216] proposed that effects on cellular reducing agent, such as thioredoxin could lead to altered redox status of the cells.

The biological effect of Se depends on the expression of Se in each chemical form [124,173,188]. Data from studies have indicated that methylselenol (MSe) is a critical metabolite in Se chemoprevention [118]. MSe is a highly reactive metabolite and therefore cannot be tested; however, precursors like MSC can generate it endogenously. MSC is a lower homolog of SeMet and could be used to test whether MSe is the form responsible for Se chemopreventive activity [119,217]. MSC is twice as active as SeMet in suppressing mammary tumorigenesis in rodents [118]. For example, at a level of 2 ppm Se in the diet, MSC consistently produces a 50% decrease in tumor formation, whereas the same dose of SeMet produces only a 20% inhibition or less. MSC inhibited clonal expansion of premalignant lesions in the mammary gland of methylnitrosourea-treated rats [120]. MSC was also found to significantly inhibit cell proliferation and the expression of cyclins D1 and A. However the expression of p27, an inhibitor of cdks was increased. In addition, MSC also increased apoptosis by almost 3-fold as well as decreasing bcl-2, an apoptosis repressor protein [120].

Selenite and selenodioxide are metabolized by GSH forming selenodiglutathione, which is then reduced to selenopersulfide anion leading to superoxide production [218,219]. Likewise, Secys metabolism by reduced GSH and/or GSH reductase into GSH selenenyl sulfide produces superoxides via redox cycling [220]. The selenite and selenocystine generation of superoxides and thiol oxidation contribute to apoptosis by opening the mitochondrial permeability transition (MPT) pore leading to release of pro-apoptotic cells [221].

Se and Hepatocarcinogenesis

The chemopreventive effects of Se have been tested on different hepatocarcinogens using different animal models in an attempt to elucidate the mechanisms involved in the protective effect of Se. Altered hepatic foci (AHF) are putative preneoplastic lesions in the liver that are used as markers for cancer effects by non-genotoxic carcinogens as well as for studying the risks posed by suspected carcinogens [222-225]. Most carcinogenesis models investigating the ability of a chemical to produce tumor nodules or lesions during the stages of initiation, promotion or progression use the initiation-promotion model usually involving partial hepatectomy or resistant hepatocyte (initiation-selection) models [226,227]. In the resistant hepatocyte model, chemicals (2-AAF, PCBs) are considered cancer promoters because their mitoinhibitory action prevent the normal hepatocytes from responding to chemical (e.g. carbon teterachloride) hepatoxicity or partial hepatectomy-induced growth stimulation; thus allowing the initiated cells that are resistant to the mitoinhibitory effect to proliferate [227,228].

Prior supplementation of Se before administering DEN significantly reduced the number of tumors in rats [229]. In a similar study using PB as tumor promoter, Se supplementation was observed to have better protection against hepatoma when given to rats before the DEN initiation phase compared with rats receiving supplementation during the promotion stage [230]. Dorado et al. [231] supplemented female rats with 4 and 6 ppm Se during the pre-initiation stage, during the promotion stage only or throughout the entire experiment (40 weeks). No Se effect was observed in relation to hepatic nodules or carcinoma incidence, and regardless of what stage the Se was given, Se supplementation did not produce protection against hepatocellular cancer [231]. An earlier similar study using lower Se dose (2 ppm Se as selenite) produced the same finding [232].

Using 2-acetylaminofluorine (2-AAF) as a promoter, Se doses of 1 and 5 ppm (as sodium selenite) administered to Fisher 344 rats were found to have no effect on the number of hepatic nodules and the volume fraction of tumor tissues during initiation and progression stages, respectively [233]. However, rats receiving 1 ppm Se during the selection phase had decreased density of liver nodules (25%) compared to the non-

selenite group (38%) and a greater decrease were seen in the 5 ppm Se group (14%). It was observed that feeding rats with 3.0 and 6.0 ppm Se as sodium selenite decreased the growth of foci induced by dimethylnitrosamine [234].

Aflatoxin B_1 (AFB$_1$) is a potent hepatocarcinogenic mycotoxin in experimental animals. Se (5 ppm) supplemented in drinking water to rats was found to inhibit altered hepatic foci induced by 1 dose of AFB$_1$ followed by partial hepatectomy and PB [235]. Baldwin and Parker [236] employed 12 doses of AFB1 followed by phenobarbital and observed that on a standard diet Se was effective in reducing foci during the promotion stage but not during the initiation phase. It was found that inhibition of AFB$_1$ induced hepatocarcinogenesis occurs mainly at the initiation phase although Se also have inhibitory effects on the progression stage of nodules to hepatocellular carcinoma [237]. Male Wistar rats fed with 0, 3 and 6 ppm Se (as sodium selenite) given in drinking water for 30 weeks were administered repeated AFB$_1$ dosing during a period of 18-27 weeks. The AFB$_1$+ Se groups had decreased number of nodules/cm2 and smaller average area of the gamma-glutathione transferase (GGT) positive foci than the Se deficient rats, but the 3 ppm group showed greater inhibitory effects than the 6 ppm group, which showed signs of toxicity. In addition, Se appeared to prevent progression of the nodules to full blown hepatocellular carcinoma even after cessation of AFB$_1$.

Glauert et al. [238] found that Se inhibited the incidence of ciprofibrate-induced altered hepatic foci in rats, which correlated with an increase in the serum and liver GPx1 activity. However, Se did not decrease the oxidative damage indices such as thiobarbituric acid reactive substances (TBARs) and conjugated dienes, indicating that increased GPx activity may have no protection against oxidative damage. Hepatoma cells injected into Sprague Dawley rat livers resulted in decreased GPx1 activity but no significant effect on oxidative stress markers, TBARs and 8-hydroxydeoxyguanosine (8-OHdG), were seen [239]. Se supplementation reduced lipid peroxide levels in tissues [240]. Se was supplemented either before initiation or during initiation and selection/promotion phases of hepatocarcinogenesis and was found to be effective in altering hepatic lipid peroxidation and antioxidant enzyme activities either in the hepatoma or in the normal liver tissues. Moreover, increased level of lipid peroxidation products and reduced levels of antioxidants, superoxide dismutase and catalase, were

observed in non-tumor bearing organs; however, these conditions were reversed to normal upon Se supplementation.

In contrast, it was shown that high Se diet (2.0 ppm as sodium selenite) given to DEN initiated rats had no effect on DEN induced 8-OHdG and no correlation was observed between GPx activity and 8-OHdG levels [241]. In fact, they found that high Se diet increased liver 8-OHdG levels; therefore, instead of protecting against DNA oxidative damage, inorganic Se supplementation may be enhancing DNA oxidative damage in vivo.

When 2-AAF was used as tumor promoter, selenite did not produce an effect on GPx1 activity [233]. The liver GPx1 activity of rats given SeMet was also shown to be either not affected or only slightly increased as a result of long term 2-AAF feeding [242]. They also observed high GSH levels in the nodules as well as the surrounding parenchyma.

Berggren et al. [243] observed that hepatic TrxR activity of rats fed with high sodium selenite (1.0 ppm) diet had 2-fold increase in hepatic TrxR activity; however, this increase was not sustained and was not accompanied by a corresponding increase in TrxR protein synthesis. They suggested that this may be caused by decreased Se incorporation leading to decrease in TrxR protein synthesis. Increased TrxR proteins in tumors of TGFa/c-myc transgenic mice was noted compared with normal liver tissues [204].

No difference in the DNA synthesis was found between severely Se deficient rats (0.01 ppm Se as sodium selenite) and Se adequate rats (0.33 ppm) [244]. On the other hand, after pretreatment with hepatopoietin or partial hepatectomy, a 3-fold increase in cell proliferation was observed in the Se-deficient rats suggesting that DNA synthesis is induced by severe hepatic stress.

CHAPTER 2: BACKGROUND ON PCBS AND CANCER

Background and Properties

Polychlorinated biphenyls, commonly referred to as PCBs, are a family of chemicals that has a basic structure consisting of 2 benzene rings and one to ten chlorine atoms placed in any of the ten available carbon atoms on the ring structure (Figure 2.1). PCBs have a chemical formula of $C_{12}H_{10-n}Cl_n$. PCBs mixtures are products of the biphenyl chlorination process, which used anhydrous chlorine in the presence of iron catalyst. The PCBs commercial mixtures are clear viscous liquids ranging from light, oily fluids to sticky resins. Commercial and environmental PCBs mixtures are present as complex mixtures of structurally-related PCBs, referred to as isomers and congeners. Isomers are congeners with the same number of chlorines, but with the chlorines in different positions. There are 209 possible congeners of PCBs in PCB commercial mixtures and each PCB congener is unique with respect to number and position of chlorines (in ortho-, para-, or meta- positions)[245]. A PCB with 4 chlorine atoms is called a tetrachlorobiphenyl, while one with 6 atoms is referred to as a hexachlorobiphenyl. More specific names are assigned to describe the exact chlorine positions on the benzene ring, e.g. 3,3',4,4'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl; these can be abbreviated as 3,3',4,4'-TCB and 2,2',4,4',5,5'-HCB, respectively. A short hand nomenclature was also devised by arranging the congeners in ascending numeric order and assigning them with "Ballschmiter" or IUPAC numbers of 1 to 209. Hence, 3,3',4,4'-TCB is referred to as PCB 77 and 2,2',4,4',5,5'-HCB is PCB 153 [246] .
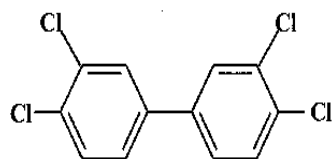
3,3',4,4' - Tetrachlorobiphenyl
PCB 77 -- Coplanar
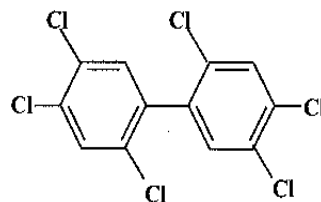
2,2',4,4',5,5' - Hexachlorobiphenyl
PCB 153 -- Non-Coplanar

Figure 2.1: Structure of PCBs. The para and/or meta positions are chlorinated in coplanar congener PCBs while non-coplanar congeners have at least 2 ortho- chlorines.

The 2 phenyl rings of PCBs can rotate about the connecting single bond. The preferred low-energy conformation depends on the degree of chlorine substitution. PCBs that have no ortho-chlorine substitution are referred to as coplanar congeners [247-249]. These so-called coplanar congeners are TCDD mimics because, like TCDD, they also bind with higher avidity to the aryl hydrocarbon (Ah) receptor [250-252]. 3,3',4,4' –TCB (PCB 77) typifies a coplanar PCB. PCBs that possess chlorine in the ortho positions are called non-coplanar or ortho-substituted PCBs. 2.2',4,4',5,5'-HCB (PCB 153) is an example of this group of congeners. PCBs, like PCB 153, that contain chlorine in para positions and at least 2 meta positions, and one ortho-position substituted with chlorine atoms were found to bind with avidity to the CAR receptor. PCBs with characteristics of both co-planar AhR agonists and CAR agonists may be mixed inducers of xenobiotic metabolism. An example is 2,3,3',4,4',5-HCB [253,254]. On the basis of the number and placement of chlorine atoms, PCBs will bind to a variety of receptors, active sites of enzymes, pockets in transport proteins, etc. and this binding ability was believed to determine their biologic effects.

The low water solubility or high lipophilicity of PCBs renders them soluble in most organic solvents, oils, and fats. PCBs have high thermodynamic stability and do not degrade easily. PCBs may be destroyed only under certain conditions by chemical, thermal, and biochemical processes such as incineration or metabolic degradation although the latter proceeds quite slowly [255,256].

PCBs Production, Uses and Distribution

The production and commercial use of PCBs began in 1929 although PCBs were first synthesized in 1881 by Schmidt and Schulz [251]. PCBs properties which make them suitable for various industrial applications include thermal stability, chemical inertness, non-flammability, high electrical resistivity or high dielectric constant, and low acute toxicity. Because of their stability under a broad range of chemical, thermal and electrical conditions, PCBs were used as oil in transformers, dielectrics in capacitors, hydraulic fluids in hydraulic tools and equipment, and heat exchange liquids. Widespread use of PCBs was found in the formulation of lubricating and cutting oils, in flame

retardants, and as plasticizers in manufacture of adhesives, sealants, carbonless copying paper, paints and varnishes, dyes, waxes and plastics [257].

PCBs were produced for industrial use as mixtures of multiple congeners and were sold under different brand names. In the United States, PCBs were produced by the Monsanto Chemical Company under the name "Aroclor", and the production groups were labeled as 1221, 1232, 1242, 1248, 1254 and 1260. The last two digits denote the percentage of chlorine contained in the mixture by weight. Other commercial mixtures of PCBs were also produced and sold in other countries under different trade names including Clophens in Germany, Phenoclors in France, Fenclors in Italy, Kanechlors in Japan, Sovol in Russia, Chlorofen in Poland, Fenochlors in Spain and Delor in Czechoslovakia [255,258,259]

The overall, worldwide production of PCBs mixtures was estimated to be approximately 1.5 million metric tons, with the United States accounting for almost a third of PCBs distributed worldwide. The US production of PCBs from 1929 to 1977 was estimated to reach about 1.1 billion pounds. Although production of PCBs ceased in North America and Western Europe in the 1970's, and in Eastern Europe and Russia in the 1990's, PCBs persist in the environment and continue to be detected as a result of discharges into waterways and landfills, inappropriate use of PCBs as extenders in agricultural formulations, leaks or leachates from existing PCBs-containing equipment, improper disposal of PCBs containing equipment, and electrical fires. In US, the PCBs pollution of the Hudson river occurred during the process of filling transformers with PCBs. Fires involving electrical transformers and capacitors in Binghampton, NY in 1981 and in San Francisco in 1983 contributed to the PCBs environmental load [255,260].

The chemical resistance and lipophilicity of PCBs contributed to their widespread, global transport. PCBs are transported by air, water, fish, and birds and can be deposited from air by rain, snow, dry fall-out and vapor-phase deposition. PCBs migrated to the lower latitudes where evaporation predominates and to the polar region where deposition predominates. However, most PCB pollutants may remain in a "sink" such as deep ocean sediments [255,261-263]. The environmental transport of PCBs is believed to cause the fairly uniform global distribution of PCBs [264,265].

As a result of their lipophilicity and relative insolubility in water, PCBs are able to bioconcentrate, bioaccumulate and biomagnify in higher trophic levels of the food chain. The reported average octanol/water partition coefficient or log K_{ow} values for PCBs isomer groups ranged from 4.7 to 8.3. The increasing number of chlorines influences the lipophilicity of a given PCB formulation [255,266]. Bioaccumulation is found to be congener dependent and may be roughly correlated to degree of chlorination [255].

PCBs and Human Exposure

The increased level of PCBs found in human serum, blood, adipose tissue, and human milk and milk fat in studies of different groups of population is attributed to several routes of human exposure to PCBs. Humans are exposed to PCBs through contaminated food, air and water, occupational exposure, and accidental exposure through food containing PCBs. Food contamination arise via food packaging, crops grown on contaminated soil, contaminated feed for animals or seafood from environmentally contaminated regions. In humans, the average PCBs levels in adipose reach ppm level, in mother's milk around 0.5 to 2.5 ppm, and in blood around ppb levels [251,255,267-269].

Associations between PCB exposure and the effects in humans have been studied in occupationally exposed populations. Workers employed in transformer and capacitor manufacturing plants are among those with long exposure to PCBs. These workers had daily contact with newly synthesized PCBs; however, aside from skin irritation and transient increase in liver enzymes, no acute or chronic health effect could be linked to PCBs [270,271]. From other studies of occupationally exposed workers, the effects of PCBs reportedly include chloracne and dermal lesions, hepatic effects (elevated liver enzymes levels and lipids in the serum, induction of drug metabolizing enzymes and hepatomegaly), decreased birth weight of children of PCBs-exposed mothers, reduced pulmonary function, and eye irritation. However, no correlation was established between PCB exposure levels and PCB-induced toxicity in the occupationally exposed populations [272-275]. In addition, increased cancer mortality among workers was not observed in association to PCB exposure [276]. On the other hand, a possible link

between PCB exposure of workers and malignant melanoma, cancer of the brain, gall bladder and pancreatic cancer was noted [277,278]. In summary, occupational studies failed to demonstrate causal association between PCBs exposure and increased risk of overall mortality or cancer mortality.

The most significant evidence of PCBs toxicity in humans was from two separate incidents in Japan and Taiwan, the so-called Yusho and Yucheng incidents. The populations involved in these incidents have been the subject of extensive PCB-related clinical and epidemiological investigations. In 1968, a mass food poisoning called Yusho (meaning "oil disease") occurred in the Kyushu province of Japan. Although the poisoning was at first believed to be caused by ingestion of a commercial brand of rice oil contaminated with PCBs (Kanechlor-400), it was later found to be due to the spectrum of PCBs and thermal degradation products of PCBs, such as polychlorinated derivatives of terphenyls, quarterphenyls, and dibenzofurans [279-281]. The poisoning, involving more than 1,850 people, caused acute toxicity characterized by a severe and persistent form of acne called chloracne and skin pigmentation alteration, irritation and increased discharge of the eyes, fever, jaundice, headache, numbness in the limbs, general fatigue and weakness, as well as liver disorder [270,280,282,283]. Some children born to exposed mothers had darkly pigmented skin, showed psychomotor delays and speech problems, and were generally small and intellectually impaired [284,285]. Exposed adults were reported to suffer from respiratory distress and major chloracne that eventually subsided after fifteen years [286].

A similar poisoning episode occurred in Taiwan in 1978. Reported as Yucheng disease, people became ill from ingestion of rice oil (Yucheng) also contaminated by heat-degraded PCBs. Involving more than 2,000 people, the Yucheng incident produced symptoms similar to those seen in the Yusho incident including reproductive dysfunction, liver and lung cancer [287-292]. Increased mortality from liver diseases besides cancer was also noted in the Yucheng patients. The toxic effects were attributed to PCBs thermal degradation byproducts, which are believed to be more toxic at lower dose levels than PCBs [282].

Low-level environmental exposure studies in Michigan, North Carolina, The Netherlands, New York, Germany and Canada among others have reported that prenatal

exposure to PCBs through consumption of sports fish may be associated with subtle neurodevelopmental effects in newborns and children [255,293]. However, methodological deficiencies and inconsistent findings present difficulties in accurately assessing effects of environmental exposure to PCBs [260].

PCBs could be transported to the Arctic by oceanic and atmospheric currents. Because of PCBs biomagnification, the populations living in the Arctic region could be exposed via the arctic aquatic food chain. Hence, monitoring of human exposure to PCBs in the Arctic (circumpolar countries) under the Arctic Monitoring and Assessment Program (AMAP) was undertaken by analyzing blood samples from mothers for PCB congeners (PCB 28, 52, 99, 105, 118, 128, 138, 153, 156, 170, 180, 183, 187) [294]. They found that due to consumption of marine mammals the levels of PCBs were higher in some arctic populations. Moreover, the PCB concentrations were noted to be at levels where there could be subtle effects on learning and the immune system. In some areas of Arctic Canada, PCBs (as Aroclor 1260) were detected in all maternal blood samples [295]. In addition, plasma results for PCBs in Inuit mothers were found to be 3.3 times higher than the Dene/Metis and 3.4 times higher than Caucasians. Similarly, the umbilical cord blood PCBs levels were higher among Inuit newborns than other ethnic groups. A study of pregnant women in the Faroe Islands, a North Atlantic island where whale meat and blubber and marine food is part of the diet, found high levels of hydroxylated PCBs and PCBs in the serum samples [296]. This study proves that PCBs could be found at high concentrations in areas which are distant from PCBs pollution sources.

The issue on endocrine disruption brought about by environmental pollutants received public attention with the release of *Our Stolen Future [297]*. Studies have found that although PCBs and its metabolites exhibit both estrogenic and anti-estrogenic activity, they have weak estrogenic potential and their potency is too small compared to the natural estrogen in the body [298,299]. Studies on association between PCB exposure and breast cancer or PCB and other endocrine effects such as miscarriage, stillbirth, fetal death, conception delay and menstrual length cycle do not support an "endocrine disruptor" role for PCBs [260,298].

The U.S. Environmental Protection Agency (EPA) has established a reference dose (RfDs) of 0.02 and 0.07µMol/kg body wt/day for Aroclor 1254 and Aroclor 1016, respectively [300,301]. RfD is a dose below which no adverse effects will occur even in the most sensitive population when exposed to the chemical over a lifetime.

PCBs Absorption, Distribution and Disposition

In humans, PCBs are absorbed mainly through ingestion of PCB-contaminated foods although dermal [262,302]. Gastrointestinal tract absorption is rapid and efficient approaching to almost [303,304]. PCBs are absorbed on a congener specific basis by passive diffusion. A high diffusion gradient and nearly complete absorption occurs when the PCB level in the gut contents (lipid basis) is much greater than the concentration in serum lipids. The predominant PCB carriers in human plasma are in the lipoprotein fraction. Due to their high lipid-water partition coefficients, PCBs tend to accumulate in lipid-rich tissues. The tissue distribution was found to be highest in adipose tissue followed by the skin, liver, muscle, and blood [251]. Absorbed PCBs through the gastrointestinal tract enters the blood circulation through the lymphatic system or through the hepatic portal system. Ingested PCBs (Aroclors 1242, 1254 and 1260) by animals were reported to be sequestered into chylomicrons which could then be transferred from the lymphatic system via the thoracic duct into the vascular system, hence bypassing the liver [305-307].

PCBs are absorbed less efficiently when administered by the dermal route. Inhalation absorption data are insufficient for estimating absorption rates. Data on lethality or decreased longevity due to acute or chronic inhalation of PCBs are not available [304].

Since PCBs are very lipophilic, they are transported in the blood plasma through carrier molecules. In addition to albumin, pre-albumin and corticosterone binding globulins, lipoproteins (VLDL, LDL, and HDL) are also associated with non-specific binding to PCBs [308-310]. PCBs from the liver are associated with new VLDLs, which are then redistributed to the LDLs and HDLs. However, once in the circulation, albumin is the major carrier of PCBs. In human plasma, it has been shown that 44% of parent PCBs and 61% of the methylsulfone metabolite were associated with the plasma fraction

that is rich in albumin [311]. From the blood, the liver and muscles act as initial depots for PCBs because of high perfusion rate and large tissue volume. With a decrease in PCBs exposure level or as PCBs are eliminated due to lactation or metabolism, the stored PCBs could re-enter the circulation. Stored PCBs in adipose tissue appears to be influenced by their binding affinity to adipose tissue and metabolism [312].

Feces was found to be a major route of excretion of PCB 77 in mice and rats [313,314]. The fecal PCB 77 reportedly came from the bile. Excreted forms in urine and feces were found to be mostly PCBs metabolites [310]. Some PCB congeners are relatively poorly metabolized and thus can remain in the body for long periods of time (months to years).

PCBs Toxicity and Metabolism

Toxic responses to PCBs are affected by sex, age, animal strain, route and length of exposure, the individual congeners contained in the PCBs mixtures and their interactions within the same family and with other chemical contaminants [251].

In rats, single-dose LD_{50} values of 4,250 mg/kg for Aroclor 1242, 1,010 to 1,295 mg/kg for Aroclor 1254, and 1,315 mg/kg for Aroclor 1260 have been reported [251]. The variation in LD_{50} values is due to differences in PCB congener composition. Moreover, the variation may be related to animal strain, age, sex, or formulation purity [304]. For example, studies found that immature rats (3–4 weeks old) are more susceptible than adults. Studies have also shown that female animals are more sensitive to PCBs toxicity than males, and the young is more susceptible than adults. This gender difference has also influenced the response to PCB-induced biochemical responses and tumorigenicity [315-318].

Coplanar PCBs have few or no ortho chlorines and have strong binding affinity for the cytosolic aryl hydrocarbon receptor [252,253]. These PCBs resemble 3-methylcholantrene and TCDD in their mode of cytochrome P450 (CYP) induction, mainly increasing the transcriptional activation of CYP1A1, CYP1A2, and CYP1B1 genes through the AhR in conjunction with the AhR/AhR nuclear translocator (ARNT) [319,320]. In addition, the liganded AhR also initiates gene activation/transcription of the UDP-glucuronyltransferase, glutathione S-transferase, aldehyde dehydrogenase enzymes

[245,321-323]. The PCBs-induced CYP1A1, CYP1A2 and CYP1B1 enzymes could catalyze the activation of pro-carcinogenic PAHs and nitro-PAHs into highly reactive electrophiles that could initiate cell transformations leading to tumor promotion [324,325]. In addition to carcinogenesis, other toxicological effects of coplanar PCBs include thymus atrophy, immunological and reproductive effects, wasting syndrome, neurotoxicity, porphyria and hepatic toxicities such as hepatomegaly [251]. The PCB congeners 3,3',4,4'-TCB (PCB 77) and 3,3',4,4',5'-PCB (PCB 126) belong to this group. PCB 77, one of the most potent CYP inducer, is among the 17 PCBs found in abundance in human tissues, has low degradation rate in vivo, and strongly binds covalently to cell macromolecules [301]. This congener has also been found to mimic estrogen action through interaction with the estrogen receptor [326].

The non-coplanar or ortho-substituted PCBs, having at least 2 ortho chlorine substituents, are another class of CYP inducers with an induction patter similar to phenobarbital (PB). Like PB, the members of this group bind to the constitutive androstane receptor (CAR) in conjunction with the retinoid X receptor (RXR) and induce CYP2B, 2C, 3A, UGT and GST enzymes [301,327]. The difference in induction responses is influenced by the positions of the chlorine substitutions [251]. Like PB, these PCBs have hepatic tumor promoting activity, produce hepatic enlargement, and have neurotoxic, cytotoxic, estrogenic as well as behavioral effects. However, compared to the coplanar PCBs, they are less acutely toxic, weak promoters of hepatic preneoplastic foci and do not produce thymic atrophy [251,328,329]. Examples of PB-type inducers are 2,2', 4,4'-TCB (PCB 47) and 2,2',4,4',5,5'-HCB (PCB 153) [252,330]. The latter is relatively resistant to metabolism and bioaccumulates in most species; hence, it is very persistent in the environment.

PCB congeners that can induce both CYP1A and CYP2B genes are referred to as "mixed type" inducers. These PCBs usually have at least 1 ortho-substituted chlorine. They can bind to either the AhR or the CAR; however, they bind to the AhR at a lower affinity than the coplanar PCB congeners [253,254]. The 2,3',4,4',5-PCB congener (PCB 118) is considered a mixed-type inducer. Some non-planar PCBs such as PCB 47 and PCB 184 have been found to induce CYP3A23 by activating its hormone response element via another nuclear hormone receptor, pregnenolone X receptor (PXR) [331].

PXR has also been demonstrated to induce xenobiotic metabolizing enzymes including some CYP3A, CYP2B6, CYP2C8, CYP2C9, CYP2C19, hydroxysteroid sulfotransferases, some isoforms of glutathione *S*-transferases and UDP-glucuronosyltransferases [332,333].

Coplanar PCBs are believed to be more toxic than the ortho-substituted PCBs. PCBs with more than one chlorine in the ortho positions have decreased potency compared with non- or mono-ortho PCBs [251]. It has been suggested that the carcinogenicity of PCBs depends on the position of the chlorines in the PCB molecule [315]. Studies have indicated that interactions between PCB congeners or groups of congeners could produce either additive, synergistic, or antagonistic effects. Highly chlorinated PCBs can enhance CYP1A1 uncoupling and microsomal oxidative stress in vivo and in vitro [334,335]. The CYP uncoupling process occurs when polyhalogenated aromatic hydrocarbons binds to the CYP active site but are not metabolized. This facilitates or allows electrons to be leaked in the cell, therefore contributing to a CYP-dependent microsomal production of ROS [334,336,337]. Shertzer et al. [338] observed that coplanar PCBs and TCDD increase the NADPH-dependent microsomal production of H_2O_2 up to 7 fold whereas ortho-substituted PCBs have no effect on H_2O_2 production.

The biotransformation of PCBs is a slow process that leads to their degradation and elimination. Biotransformation starts with the oxidation of PCBs by hepatic microsomal enzymes, CYP1A and CYP2B to hydroxylated metabolites [339,340]. The arene oxide pathway is the major route for hydroxylation while direct oxidation without arene oxide formation can occur as a minor pathway [341-344]. The rate of hydroxylation depends on the structure of the PCB molecule. Congeners that lack unsubstituted adjacent meta-para carbon atoms like 3,3',5,5'-TCB are more slowly metabolized compared with congeners that possess at least one pair of adjacent carbon atoms like 2,2',4,5,5'-PCB [254,345]. For congeners without unsubstituted adjacent carbon atoms, the number of chlorine molecules could affect the rate of metabolism. The rate of metabolism of 3,3',5,5'-TCB is five times that of 2,2',4, 4',5,5'-HCB [346].

The metabolism of arene oxides can proceed in different ways: spontaneous isomerization into phenols, hydration by epoxide hydrolase to a trans-dihydrodiol followed by rearomatization to catechols, conjugation to glutathione (GSH) or bind to

DNA, RNA or protein. The catechols could undergo methylation in the presence of catechol o-methyl transferase to produce mono-methoxy phenols. The phenols could undergo further oxidation to form dihydroxy or trihydroxy metabolites [347]. The dihydroxy metabolites of lower chlorinated PCBs congeners through rearrangement of an epoxide or two separate hydroxylations and further oxidation catalyzed by peroxidases and/or prostaglandin synthetase H, could form quinones, which could bind nucleophilic sites on proteins, DNA or RNA to form adducts [348-350]. The catechols and hydroquinones formed can also be oxidized to semiquinone or quinone metabolites 1996. In addition, the quinone metabolites could redox cycle and generate superoxides resulting in oxidative damage [348]. Some PCB-quinone metabolites could deplete GSH and inactivate Topoisomerase II, a sulfhydryl macromolecule in vitro [351]. Reactive metabolites of coplanar PCB 77 were noted to bind covalently to rat hemoglobin [352]. This interaction with erythrocytes is believed to be the cause for the persistent presence of PCB 77 metabolites in blood in vivo.

The oxidized PCB metabolites are conjugated by endogenous compounds like UDP-glucuronic acid (UDPGA), 3'-phosphoadenosine-5'-phosphosulfate (PAPS) or glutathione (GSH) to form polar products. The glucuronide and sulfate conjugates are mainly excreted in urine; however, some are excreted into the bile. The glucoronidases and sulfatases hydrolyze the conjugates releasing the PCB metabolites, which can then enter into enterohepatic circulation. The GSH conjugates are excreted mainly through the bile. Some GSH conjugates can be metabolized by the gut flora or by tissue β-lyase to form thiols and upon further oxidation to thioethers [353]. The thiols can undergo methylation by S-methyltransferase and S-adenosylmethionine to form methyl sulfides, which can be further oxidized into methylsulfones [354].

PCBs and Hepatocarcinogenesis

Epidemiological studies on Yusho and Yucheng victims showed an association between increased mortality from liver cancer and PCBs. The studies on occupationally exposed workers however did not provide conclusive evidence that PCBs cause cancer in humans [304]. Studies in animals provided sufficient evidence that PCBs are animal toxicants and carcinogens and convinced the world scientific community and regulatory

agencies including the U.S. Environmental Protection Agency and the International Agency for Research on Cancer (IARC) [258] to categorize PCBs as probable carcinogens to humans [355]. In addition to EPA and IARC, the National Toxicology Program and American Governmental Industrial Hygienists (ACGIH) [356] have concluded that sufficient evidence exist to classify PCBs as known animal carcinogens. Recently however, EPA lowered the cancer slope factor for PCBs indicating that EPA considered the carcinogenic potential of PCBs to be lower than its previous assessment.

PCBs are carcinogenic in rodents, and PCBs mixtures and individual congeners were found to promote liver tumors following initiation with various genotoxic agents [258,357]. Diethylnitrosamine (DEN) is the most commonly used initiating agent in promotion studies of PCB in rodent livers. Other initiating agents used include N-2-fluorenylacetamide (2-FAA), N-nitrosamine, N-nitrosomorpholine (NNM), 2-nitropropan, N-ethyl-N-hydroxyethylnitrosamine (EHEN) and aflatoxin B1 (AFB1).

PCBs tumorigenicity in rodents was first reported in the 1970's by Nagasaki and Ito [358,359]. Nagasaki [360,361] found that mice developed hepatic tumors when exposed to PCBs (500 ppm Kanechlor-500) added to the diet. Ito demonstrated that PCBs (Kanechlor-500) coadministered with a known hepatic carcinogen, benzene hexachloride (BHCs), produced higher incidence of nodular hyperplasia and hepatocellular carcinoma compared with BHCs alone. PCBs (Kanechlor-500) administered to rats following initiation with DEN produced a significant increase in tumors, however, an acceleration of tumor growth was not observed.

Aroclor 1254 (100 ppm in the diet) administered to rats for 18 weeks following initiation with DEN significantly increased the incidence of hepatocellular carcinomas, whereas Aroclor 1254 alone did not induce tumors [362]. Using a modified protocol involving a partial hepatectomy (PH) following initiation by 2-FAA, it was demonstrated that the co-administration of 2-FAA and PCBs (500 or 1000 ppm) in male F344 rats significantly increased the number and total area of hyperplastic nodules when compared to 2-FAA or PH alone, or PCBs plus PH [363]. Using EHEN as initiating chemical, PCBs (0.05% in diet) administered for 32 weeks significantly increased the incidence of hepatocellular carcinomas in male F344 rats [364]. In this study removing the polychlorinated dibenzofuran (PCDF) impurity from the PCBs did not change the lack of

significant effect on the tumor promoting activity of Aroclor 1254 when administered alone.

In earlier tumorigenesis studies rats were exposed to PCB mixtures for longer periods, an average of 1 to 2 years. To shorten the assay time, current tumor promotion protocols use preneoplastic altered hepatic foci (AHF) as markers of tumor promoting activity [224,365-367]. AHF biochemical markers include gamma-glutamyl transpeptidase (GGT positive), reduced ATPase (ATPase negative) and placental glutathione-S-transferase (pGST positive).

Using GGT-positive foci as a marker, Pereira [368] demonstrated that a single dose of Aroclor 1254 (500 mg/kg) promoted GGT-positive foci in rat liver after a 2/3 partial hepatectomy (PH) and initiation by DEN. Pelissier [369] tested the promoting activity of PCB mixture Phenoclor DP-6 on male Sprague-Dawley rats fed with a diet containing 50 ppm Phenoclor DP-6 for 11 days following initiation with aflatoxin B1 (AFB1). The number and volume of GGT-positive foci was increased in the PCB group [370].

The tumor promoting activity of PCBs has also been demonstrated in weanling rats. Aroclor 1254 promoted tumors in suckling Swiss mice when the suckling offspring of Aroclor 1254 treated (i.p.) pregnant mice were administered N-nitrosodimethylamine [371]. Although treatment with N-nitrosodimethylamine alone produced liver tumors, PCB significantly increased the incidence of liver tumors at the age of 18 months. Oesterle and Deml [372] showed the tumor promoting activity of Clophen 50 in weanling Sprague-Dawley rats using N-nitrosamine as initiator. Clophen 50 (100 mg /kg of body weight, once a week for 1-7 weeks) following initiation produced an increased number and total volume of ATPase-deficient and glycogen- and GGT-positive altered hepatic foci. Clophen 50 alone caused few foci. In another study they noted a dose-dependent tumor promoting activity of Clophen A50 in weanling female Sprague-Dawley rats following initiation with DEN [373]. Different levels of Clophen A50 (0.1, 0.5, 1.0, 5 and 10 mg/kg of body weight, three times per week for 11 weeks) were given to rats. The lowest observable effective dose of Clophen A50 was 1 mg/kg body weight.

Gender is believed to affect PCBs carcinogenicity. It was noted that 1200 mg Kanechlor 400 given to Donryu rats for at least 57 weeks produced hepatic adenomatous

nodules in female rats, but none was seen in the male or control rats [374]. In another study, Aroclor 1260 fed to Sprague-Dawley rats, 95% female rats had neoplastic nodules compared to 15% of males [375]. The susceptibility of the female rats was attributed to differences in metabolism or difference in the levels of sex hormones. Likewise, it was observed that PCBs had more potent tumor effect in female rats compared to male rats [376]. Clophen A 50 (50 or 100 mg/kg body wt/week for 7 weeks) administered to Sprague-Dawley rats following initiation by DEN produced a significant increase in the number and volume of ATPase-deficient and GGT-positive altered hepatic foci (AHF), both of which were higher in female than male rats. Male and female Sprague-Dawley rats were treated with Clophen A50 (10 mg/kg body weight, twice a week for 8 weeks) following initiation with different doses of 2-nitropropane vapors [377]. Female rats exhibited an approximately four times higher incidence of ATPase-deficient foci than male rats. The study by Mayes et al. [315] demonstrated a strong gender difference in PCB-induced liver cancer. Male and female Sprague-Dawley rats were administered 4 different Aroclors (1016, 1242, 1254 and 1260) mixed in rodent chow for 24 months. A significant increase in neoplastic hepatic lesions was induced by all the Aroclor mixtures in the female rats, whereas only Aroclor 1260 induced a significant response in the male rats. This gender effect on the incidence of neoplastic lesions was partly explained by the increased feed intake of the female rats [315].

The role of specific PCB congeners in liver carcinogenesis has also been studied in rats and mice. It has been suggested that the chlorine position in the PCB molecule could determine carcinogenicity [315,378]. Coplanar PCBs investigated so far as well as a number of non-coplanar or ortho-PCBs congeners have been identified as tumor promoters in rat liver [357,379,380].

Different tumor promotion studies were reviewed involving coplanar PCB-77 and di-ortho PCB 153 using various AHF markers and different initiation-promotion protocols [357]. All studies demonstrated tumor promotion activities in rats [328,381-388].

Kobusch et al. [386] administered PCB-77 (five i.p. injections, 50 mg/kg body weight, every 3 days) to B6C3F1 mice following initiation with NNM and observed that the number of G6Pase-negative and positive foci was decreased while the mean volume

of foci was increased. The effect on the mean volume is suggested to be the result of an increase in the percentage of large sized foci. Both 2,2',5,5'-TCB (PCB-52) and 2,2'4,4'-TCB (PCB-47) (100 ppm in diet for 27 weeks) have also been shown to promote GGT-positive foci after initiation by PH and DEN [389].

Interactive effects between different PCBs congeners could produce effects on tumor development that could be additive, synergistic, or antagonistic [255]. When the separate and combined effects of non-coplanar 2,5,2',5'-tetrachlorobiphenyl (PCB-52) and PCB-77 were examined, the two PCBs together synergistically increased both the number and volume of PGST-positive and ATPase-negative focal lesions whereas PCB-77 did not increase the number or volume of AHF and PCB-52 increased the volume but not the number of AHF [381].

PCB-77 and PCB-153 promoted ATPase-deficient focal lesion in female Wistar rats initiated with DEN [390]. PCB-77 was found to be more potent than PCB-153, even though PCB-153 is a poorly metabolized congener, hence, has longer half-life in the body compared to PCB-77. The interactive effects between PCB-77 and PCB-153 was studied [382]. Each PCB was given to rats at a dose of 300 µmol/kg every two weeks for four injections, or the two PCBs at 150mol/kg each for, following a single dose of DEN. The co-administration of PCB-153 decreased the volume and number of foci induced by PCB-77. Using the same protocol, rats were initiated with a single dose of DEN before administering 4 i.p. injections of PCB-77 (100 or 300 µmol/kg) or PCB-153 (100 or 300 µmol/kg), or both PCBs (100 µmol/kg each) every two weeks [391]. Both PCB-77 and PCB-153 increased the number and volume of PGST-positive foci. The co-administration of PCB-153 with PCB 77 produced antagonistic effects.

PCB-153 (1000, 5000, or 20,000 µg/kg/week s.c. for 20 weeks) enhanced GGT-positive and PGST-positive focal lesions after initiation with PH and DEN [379]. PCB-153 has also been shown to promote PGST-positive focal lesion after initiation with DEN only [382,391]; the dose of PCB-153 used in both studies was 4 i.p. injections of 300 µmol/kg body weight every other week.

Haag-Grolund and co-workers [388] examined the interactive effects between a coplanar PCB (PCB-126), a mono-ortho substituted PCB (PCB-105), and PCB-153. When initiated rats were fed 15 dose combinations of PCBs for 20 weeks, an additive

effect was observed between PCB-105 and PCB-153. However, antagonistic effects were observed between PCB-126 and PCB-153 and between PCB-126 and PCB-105. This antagonistic effect between PCB-126 and PCB-153 was confirmed when female F344 rats treated with DEN followed by PH were given different doses of PCB126 or PCB-153, or the combination of two PCBs 3 times weekly for 6 weeks [392]. Moreover, the two PCBs produced an antagonistic effect in all dose combinations. PCB 126 and PCB 153 individually produced a dose dependent increase in the number and volume of PGST-positive foci.

Mechanism of PCBs-induced tumor promotion

Liver carcinogenesis can be separated into at least three consecutive phases: initiation, promotion, and progression [366,393]. Initiation results from an irreversible mutation of genes involved in genomic stability, which could be due to a chemical, physical, or microbial agent [394]. In the promotion stage, the initiated phenotype is fully expressed and results in a clonal expansion of the initiated cells, which is a reversible process. In this stage, putative preneoplastic cells or altered hepatic focal (AHF) lesions are observed [366,393,395]. The final stage of carcinogenesis is the progression phase where neoplasm develops into a benign or a malignant cancer. Tumor promoters are non-genotoxic compounds that could facilitate growth of hepatic focal lesions by several proposed mechanisms including cell proliferation and apoptosis [396-399].

Many short term tests for genotoxicity indicate that PCBs are only weakly genotoxic, if at all [400]. However, the slow rate of metabolism of PCBs in general diminishes the likelihood that reactive species will be produced in a short time frame in sufficient quantity to produce a positive finding. The negative results have led many to conclude that PCBs have little if any genotoxic potential [401]. However, studies show that PCBs mixtures are complete carcinogens in rodents [258,315,402]. On the other hand, other studies have indicated a lack of concrete evidence indicating that PCBs mixtures could act as tumor initiator in liver [383]. Pereira [368] showed that Aroclor alone did not initiate GGT-positive foci. Deml and Oesterle [403] demonstrated that Clophen alone resulted only in a small number of foci, which could have been "spontaneous" foci or foci initiated by "contaminants" in the PCB mixtures or diet.

However, in some lower halogenated PCBs, possible tumor initiating activity is suggested [404]. Using a modified Solt-Farber protocol, the potential initiating activity of lower chlorinated PCBs (PCB3, PCB12, PCB38, PCB15, PCB52, and PCB77) was investigated. Among the PCBs tested, PCB3, PCB15, PCB52, and PCB77 significantly increased the number of GGT-positive foci per cm^3 of liver and per whole liver. Only PCB3 and PCB15 increased the volume fraction of GGT-positive foci. Lower chlorinated PCBs are better candidates as initiators since they are metabolized at a much faster rate compared to higher chlorinated PCBs [405].

It is generally known that PCBs could act as tumor promoters based on 2-stage liver carcinogenic assays and in vitro studies; however, their mechanism of action has not been clearly elucidated. PCBs mixtures and congeners have been shown to promote tumors by increasing the number of tumors or by inducing clonal expansion of initiated cells. Several mechanisms by which PCBs exert their tumor promoting activities have been proposed including inhibition of apoptosis and induction of cell proliferation leading to growth of hepatic focal lesion, effects on Vitamin A metabolism, effects on intercellular communication, and oxidative damage [335,357,391,406,407]. Moreover, PCBs have been shown to lower glutathione peroxidase activity, hepatic selenium and Vitamin E levels [317,369,408-413].

Oxidative stress occurs when the production of free radical and other oxidants exceeds the capacity of intracellular antioxidants. Reactive oxygen species (ROS) released as a by-product from cytochrome P-450 could contribute to lipid peroxidation and other forms of oxidative damage. Other sources of oxidative stress may include other induced enzymes or the repression of antioxidants or of antioxidant enzymes. Increased oxidative stress is one mechanism of PCB-induced tumor promotion [357]. Lipid peroxidation products such as malondialdehyde (MDA) and hydroxyl radical could form DNA adducts, react with cellular membranes and proteins and produce cellular effects [414-417]. In addition, oxidative stress blocks gap junction intercellular communication, an important mechanism of tumor promotion and cell proliferation [418]. Lipid peroxidation studies on Kanechlor 500, Aroclor 1248 and congener mixtures using hepatic thiobarbituric acid reactive substances (TBARS) as lipid peroxidation indicators found that PCBs increased TBARS in the liver [419,420]. Employing MDA as indicator,

Dogra et al. [421] found that Aroclor 1254 and PCB congeners PCB-77, PCB-114 and PCB 153 (300 μmol/kg each) increased MDA concentrations 30 days after administration. The increase in MDA concentrations resulting from PCB-77 and PCB-153 administration in rats was confirmed by Fadhel et al. [335] using 150 μmol/kg of each PCB. On the other hand, another study found that PCB-153 (400 μg/kg p.o.) given for 3 days did not affect hepatic MDA whereas another coplanar PCB, PCB 126, produced increased hepatic MDA concentration

PCBs, like some xenobiotics are believed to produce cancer due to excess (ROS) generation [422,423]. ROS may be generated during the oxidative metabolism of PCBs [424,425]. The ROS generated during PCB metabolism and PCB-initiated redox cycling may produce oxidative DNA lesions such as 8-oxo-deoxyguanosine [426]. Moreover, metabolic activation of PCBs to reactive species has been demonstrated by the covalent binding of PCBs to protein, including nuclear protein, as well as to RNA, DNA, and hemoglobin [427,428]. In addition, ROS could induce changes in gene expression such as the overexpression of c-fos, c-jun, and c-myc [429] or could stimulate the transcription factors activator protein 1 (AP-1) and nuclear factor-κB (NF-κB), which could lead to enhanced cell proliferation [430,431]. ROS has also been shown to stimulate protein kinase C (PKC), another regulator of hepatocyte proliferation [432].

In vivo and in-vitro studies have found that PCBs mixtures (Aroclor 1254, 1260) inhibit intercellular communications between hepatocytes [418,433]. The same effect on intercellular communication was exhibited by many PCB congeners including PCB-52, PCB-77, PCB-114, and PCB-153; however, PCB 169 was not effective [434-436].

CHAPTER 3: EFFECTS OF THE PCB 3,3',4,4'-TETRACHLOROBIPHENYL ON SELENIUM AND GLUTATHIONE PEROXIDASE IN RAT LIVER

Abstract

Polychlorinated biphenyls (PCBs), like 3,3',4,4'-tetrachlorobiphenyl (PCB 77), induce drug metabolism and promote oxidative events within the cell. Selenium (Se), and selenium-dependent enzymes, e.g. glutathione peroxidase (GPx1), mediate against oxidative damage. The goal of the present study was to determine the influence of PCB 77 on hepatic total Se and GPx1. To examine these parameters, a time-course study using male and female Sprague Dawley rats, receiving a single i.p. injection of PCB 77 (300 µmol/kg) was carried out. Rats were killed 6 hours, 12 hours, 1 day, 4 days, 1 week, 2 weeks, or 3 weeks later. In male and female rats, hepatic microsomal cytochrome P-450 1A1 activity (ethoxyresorufin O-deethylase) was increased at all time points, 6 hours through 3 weeks. Only female rats showed a significant decrease in GPx1 activity and in hepatic total Se levels. These findings point to potential markers of PCB exposure and oxidative stress that, in the rat, are seen in both genders, or are evident in male or in female rats.

Introduction

Polychlorinated biphenyls (PCBs) are recognized, ubiquitous environmental contaminants. High lipophilicity, resistance to a wide variety of physical, chemical and thermal agents, and ability to bioaccumulate and bioconcentrate contribute to PCBs' persistence and broad distribution [251,437]. PCBs that were produced for commercial use are mixtures of congeners, which exhibit varying toxicities depending on their structure. A group of PCB congeners, called "coplanar" PCBs, or "dioxin-like" PCBs are aryl hydrocarbon receptor agonists and induce cytochrome P450 1A1 (CYP 1A1). PCB 77 (3,3',4,4'-tetrachlorobiphenyl) is an AhR agonist and efficaciously induces CYP 1A1 [438]. This enzyme in the presence of PCB 77 or a metabolite may generate ROS via an uncoupling of the catalytic cycle and a partial reduction of oxygen [336,337]. In addition, PCBs may be substrates for the induced cytochrome P-450. The catalyzed oxidation of lower chlorinated biphenyls gives rise to mono- and di-hydroxy metabolites. The latter can auto-oxidize or can be enzymatically oxidized to semiquinones and/or quinones [349]. Some PCB-quinones can undergo redox cycling leading to formation of reactive oxygen species, thus becoming a source of oxidative stress [348].

Selenium (Se) is an essential trace element and a component of twenty-five known selenoproteins, almost half of which are reported to play a role of antioxidants [46,212]. These include the glutathione peroxidases and thioredoxin reductases that are known to protect against oxidative damage. In addition to its role as antioxidant, selenium has been shown to have protective effect against several types of cancer in human and animal models [20,128,169,170,188,439,440]

Of the known selenoproteins, selenium-dependent glutathione peroxidase (GPx) has been used as a bio-marker of selenium status in human and animals [197,441]. GPx1 confers protection from oxidative damage by reducing hydrogen peroxides and organic peroxides [63,442-444]. Together with vitamin C and vitamin E, GPx1 provides antioxidant defense against reactive oxygen species and free radicals.

Exposure to environmental contaminants has been shown to lead to a Se deficient status. For example, selenium tends to form inert metal selenide complexes with mercury or methyl mercury, but this protective effect results in decreased bioavailable selenium for selenoprotein synthesis [52,445]. PCBs produce reactive oxygen species like

hydrogen peroxides, which in turn are detoxified by GPx. We showed earlier that PCBs reduce the activity of cytosolic GPx (GPx1) and recently that gene expression of GPx1 was also diminished [317,411]. A reduction in GPx1 activity was also seen in male Sprague-Dawley rats treated with a mixture of tetrachlorobiphenyls [406]. Treatment of male mice with coplanar PCBs, PCB 77 and PCB 126, also led to a decrease in GPx1 activity [446]. An investigation of individual congeners that could possibly contribute to the effects on Se and GPx indicated that PCB 77 treatment of Sprague-Dawley male and female rats produced a significant lowering of hepatic Se levels and a concomitant decrease in the GPx1 activities compared to other PCB congeners, PCB 3, PCB 4 and PCB 153 [317,411]. When combined with PCB 77, PCB153 produced the same effect as PCB 77.

In the present study, we determined the influence of PCB 77 (3,3',4,4'-tetrachlorobiphenyl) on hepatic selenium level and glutathione peroxidase including the induction of cytochrome P450 1A1. We proposed that exposure to PCB 77 produces oxidative stress-related changes in selenium and anti-oxidant activity of GPx1 in the liver. To examine these parameters, a time-course study using male and female Sprague Dawley rats receiving a single i.p. injection of PCB 77 (300 μmol/kg) was carried out. Rats were sacrificed 6 hours, 12 hours, 1 day, 4 days, 1 week, 2 weeks or 3 weeks after PCB treatment.

Material & Methods

Chemicals

PCB 77 (3,3',4,4'-tetrachlorobiphenyl) was prepared and characterized as described [411]. All other chemicals were obtained from Sigma-Aldrich Chemical Co. unless otherwise indicated.

Animals

All animal experiments were conducted with approval from the University of Kentucky Institutional Animal Care and Use Committee. Male and female Sprague-Dawley rats (150-174 g) (Harlan Sprague-Dawley, Indianapolis, IN) were housed in groups of 4 per cage. The rats were maintained at a room temperature of 22°C with a 12 hour light-dark cycle. Unrefined rat chow (Purina rodent laboratory chow, Purina Mills, St. Louis, MO) and water were given *ad libitum*. Rats received one i.p. injection of PCB 77 (300 μmol/kg body weight) or vehicle (stripped corn oil, Acros Chemical Company, Pittsburgh, PA), and were sacrificed at different time points: 6 hours, 12 hours, and 1, 4, 7, 14 and 21 days after injection. The tissues were excised and were either processed immediately (see below) or were quickly frozen in liquid nitrogen and stored at -80°C.

Liver homogenates, microsome and cytosolic fractions preparations

The 100,000xg cytosolic (glutathione peroxidase activity) and microsomal fractions (cytochrome P450 1A1 activity) were prepared as follows. A portion of the liver was homogenized in 0.25 mole (M) sucrose/0.1 mmol ethylenediaminetetraacetic acid (EDTA), then centrifuged at 10,000xg for 20 minutes. The supernatant was then centrifuged at 100,000xg for one hour, as described [447]. Protein concentrations were determined using the Lowry [448] method.

Measurement of CYP1A1 activity

Cytochrome P450 1A1 was estimated in hepatic microsomes by measuring the ethoxyresorufin deethylase (EROD) activity as previously reported [447]. Briefly, a mixture of 0.1M Tris (pH 7.4), 0.5mmol ethoxyresorufin, dissolved in DMSO) and microsomes (10ug/μl) was incubated in a shaking water bath (37°C) for 2 min. For the 0

time point, 1 ml aliquot was taken and added to 1 ml of methanol. To start the reaction, a NADPH regenerating system (0.1M β-NADPH, 0.5M G-6-P, 0.3M magnesium phosphate (pH 7.4) was added to the mixture and incubated in the water bath for 10 min. One ml methanol is added to a 1 ml aliquot of the reaction mixture to stop the reaction. The samples were read against a resorufin standard curve at 589 nm excitation and 556 nm emission using a Shimadzu RF-5301PC spectrofluorophotometer. The CYP1A1 activity is expressed as nmol Resorufin/mg protein/min.

Measurement of Se-dependent glutathione peroxidases

The glutathione peroxidase activity of the cytosolic fraction was determined using the method of Paglia and Valentine [199] as modified by St. Clair and Chow [449]. Briefly, a reaction mixture of 23.6 mg EDTA, 6.5mg sodium azide (NaN3), 16.7 mg NADPH, 30.7 mg GSH, 100 units glutathione reductase in 100ml 50 mmol Tris-HCl (pH 7.6) was prepared. In a quartz cuvette, 5μl 1:10 diluted cytosol (diluted with Tris-HCl) and 945μl reaction mixture was preincubated for 1 min at 30°C. 30% H_2O_2 is added into the reaction cuvette and the decrease in absorption was monitored spectrophotometrically (Shimadzu MPS-2000 spectrophotometer) for 1 minute at 340 nm. Enzyme activity is expressed as nmoles NADPH/min/mg protein.

Sample preparation for selenium analysis

Microwave digestion was used to permit decomposition of the sample using nitric acid only [450]. Approximately 0.5 g of wet liver tissue samples were measured into an Ultimate Digestion Vessel (UDV) and 2 ml nitric acid (J.T. Baker) were added to each sample. The vessels were placed in a CEM MDS-2000/2100 series microwave digester for high-pressure closed vessel microwave digestion for 45 minutes. The liver tissue acid digests were poured into tubes and distilled water was added to bring sample volume to 10 ml.

Total selenium determination

Selenium concentrations of liver tissue acid digests were determined using a Varian Spectra 300/400 Zeeman graphite furnace atomic absorption spectrometer (GFAAAS) equipped with a Varian Ultra AA selenium lamp and Zeeman GTA 96 plus autosampler. Briefly, commercial inorganic selenium atomic absorption standard reference material (1020 μg Se/ml of 1 wt.% HNO3 was used to prepare selenium standards for the calibration curve. Samples and standards placed in pre-labeled cups were diluted 1:4 with a 10% Triton X-5% citric acid reagent (1:5 v/v). The blank was prepared using Triton X-Citric acid reagent. The GFAAS was set at 196 nm wavelength and 2.0 nm slit width, and was programmed to make duplicate determinations per sample. The blank and standards were analyzed at the start of each run. A blank is analyzed after every 10 samples. Tissue Se concentration is reported in μg/kg wet tissue.

Statistical analysis

The mean values were compared by analysis of variance and Scheffe's test to determine the statistical significant differences.

Results

After a single bolus dose of PCB 77, increases in liver weight were observed in male rats starting at four days post treatment and continued until the week two time point. By comparison, female rats treated with PCB 77 showed increased liver weight at the earlier one day time point, that continued through the third week time point (Table 3.1). In contrast, body weight was not increased, but actually decreased in both male and female rats treated with PCB 77 at later time points. Body weight loss at no time point exceeded 10 % of the control value (Table 3.1). Examination of the ratios of liver to body weight shows a transient increase at 6 hours in both male and female rats, and then a consistent increase from day 1 through the two week time point (males) and the three week time point (females) (Table 3.1).

PCB 77 is an efficacious inducer of microsomal cytochrome P450 1A1. The associated enzyme activity, ethoxyresorufin O-deethylase, was statistically significantly increased over controls, in both male and female rats at all time points from 6 hours through 3 weeks (Figure 3.1).

Selenium-dependent GPX activity was significantly depressed in the female rats starting at the 4 day through the 3 weeks time point (Figure 3.2). In male rats, GPX activity was decreased at the later time points, but this decrease was statistically significant only at the 4 day time point (Figure 3.2). It is possible that the large variance in the male groups may have masked the effect of PCB 77 on the hepatic GPX levels of male rats.

Examination of the total selenium levels in PCB 77-treated animals showed a significant decrease in hepatic selenium levels in female rats starting at the 1 day time point and continuing until the 3 week time point (Figure 3.3). On the other hand, in male rats no significant effect was observed on the hepatic selenium concentration in PCB 77-treated male rats at any time point, in comparison to the control (Figure 3.3).

Table 3.1: Body weight, liver weight, and liver/body weight ratios. Results expressed as means ± SE with n = 4 rats receiving 300 µmol/kg/injection of PCB 77. * signifies a statistically significant change as compared to equivalent gender control.

	Body Weight (g)		Liver Weight (g)		Liver/Body Ratio	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Control	313.5±3.0	222.0±1.8	12.9±0.5	8.3±0.2	0.041±0.002	0.037±0.001
6 hours	307.8±7.2	222.5±1.5	14.6±0.1	10.1±0.4	0.047±0.001*	0.045±0.002*
12 hours	297.5±4.7	219.5±0.9	13.3±0.4	9.0±0.2	0.045±0.001	0.041±0.001
1 day	295.5±4.6	219.8±5.0	13.6±0.1	10.6±0.4*	0.046±0.001*	0.048±0.001*
4 days	310.5±5.9	218.5±3.3	16.4±0.7*	11.6±0.5*	0.053±0.002*	0.053±0.002*
1 week	311.0±8.8	209.5±3.5*	17.6±1.1*	11.8±0.9*	0.056±0.002*	0.056±0.003*
2 weeks	301.0±1.2	204.0±4.0*	16.6±0.4*	11.9±0.6*	0.055±0.001*	0.058±0.002*
3 weeks	286.3±4.6*	211.8±4.0	13.2±0.3	10.7±0.4*	0.046±0.001	0.051±0.002*
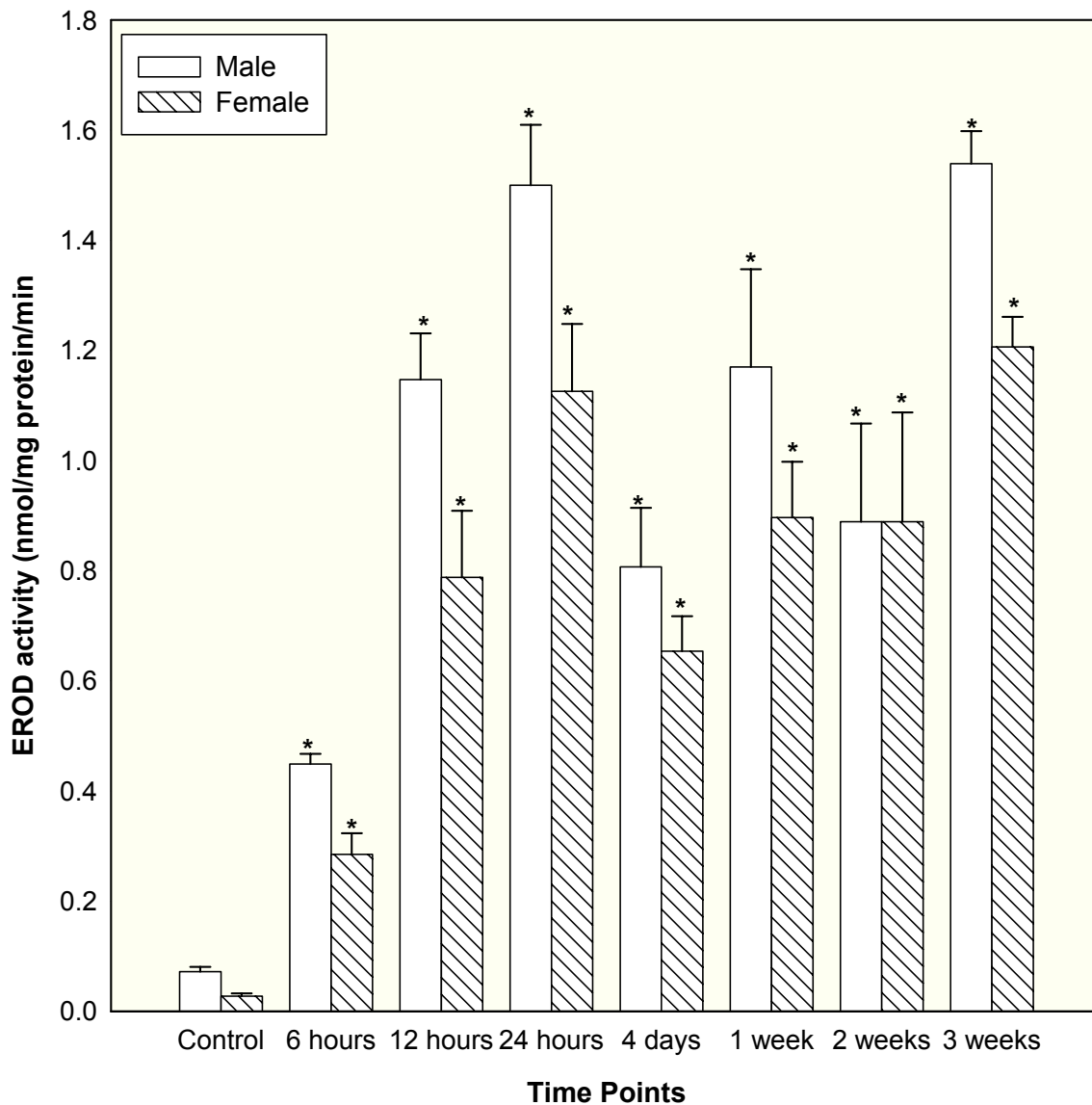
Figure 3.1: Ethoxyresorufin O-deethylase activity. Results are expressed as nmol/mg protein/min, (means ± SE with n = 4) in rats receiving 300 μmol/kg/injection of PCB 77. * signifies a statistically significant change as compared to equivalent gender control.
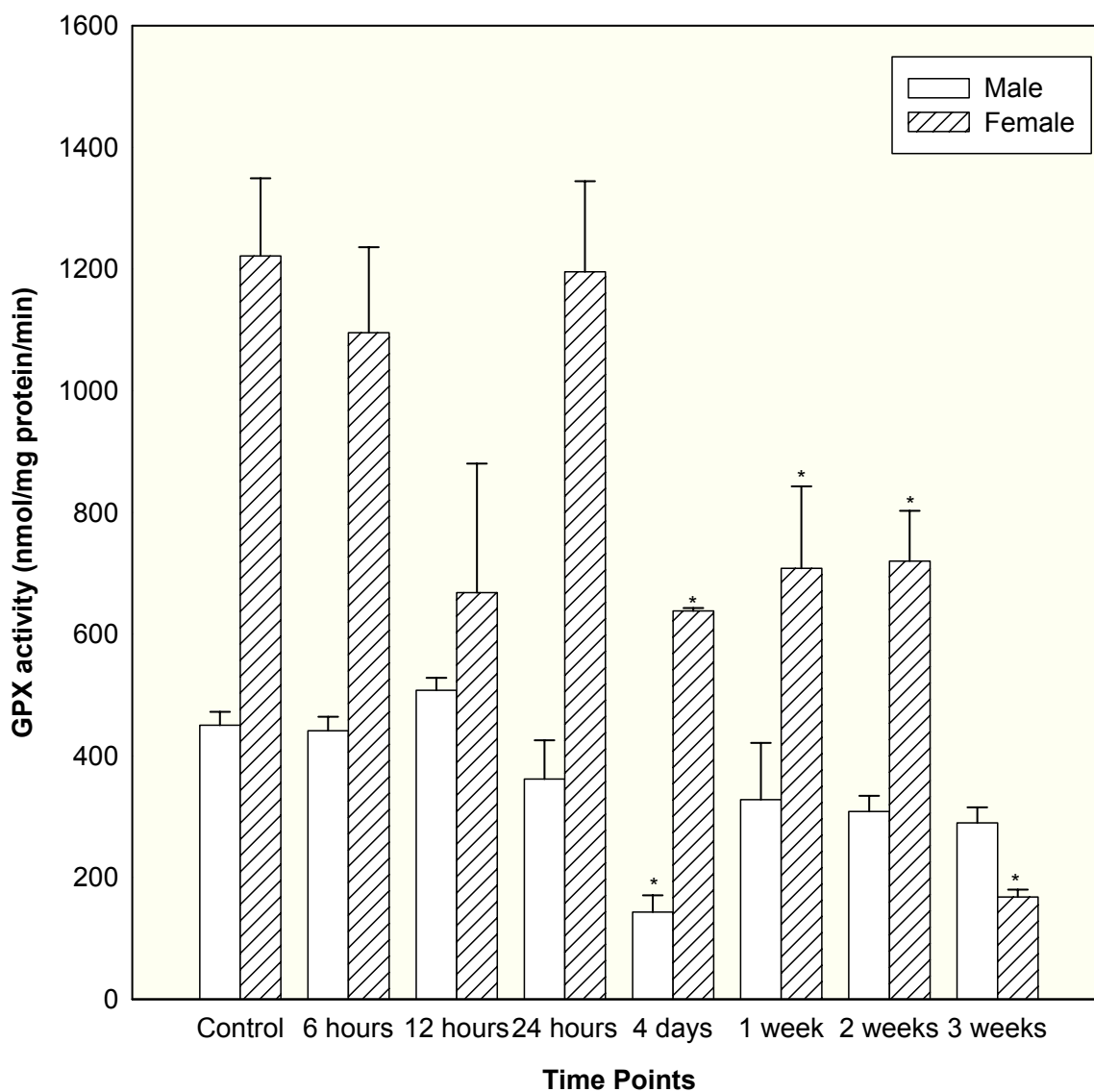
Figure 3.2: Selenium-dependent glutathione peroxidase activity. Results are expressed as nmoles/minute/mg protein, (means ± SE with n = 4) in rats receiving 300 μmol/kg/injection of PCB 77. * signifies a statistically significant change as compared to equivalent gender control.
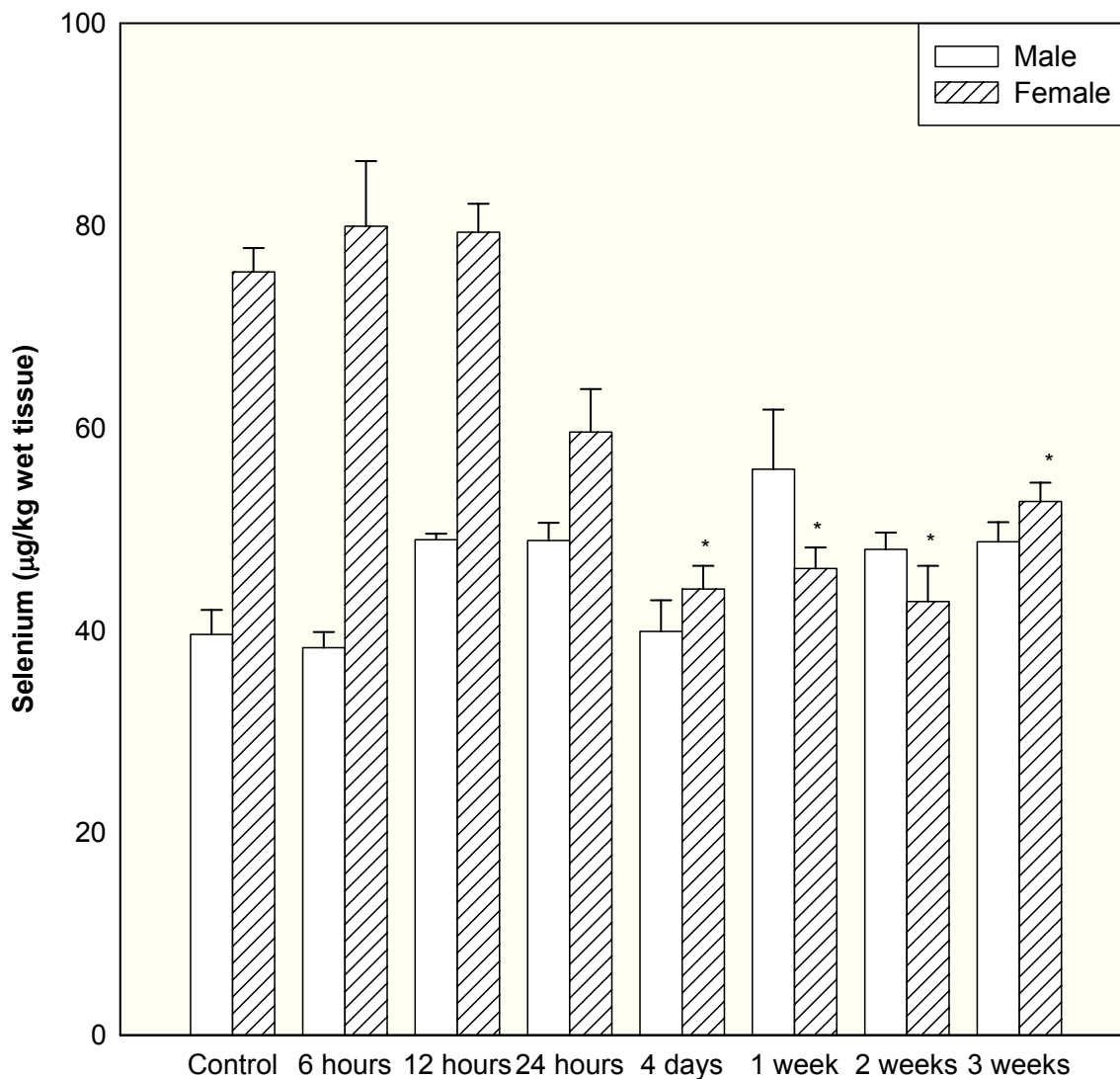
Figure 3.3: Hepatic selenium. Results are expressed as µg Se/ kg wet liver, (means ± SE with n = 4) in rats receiving 300 µmol/kg/injection of PCB 77. * signifies a statistically significant change as compared to equivalent gender control. Se concentration was analyzed using the Zeeman-GFAAS method.

Discussion

PCB 77, a "co-planar" PCB, was chosen for this study for several reasons including; it is a PCB congener found in Aroclors 1242, 1248 and 1254 although at low levels, it is an agonist of the AhR, thus it can induce CYP 1A1 and 1A2 efficaciously [252,262,335,451,452]. PCB 77 has been found to enhance hepatic lipid peroxidation and indicators of ROS and oxidative stress [336,337,350,453]. PCB 77 caused increased DNA binding activity of the oxidative stress-induced/related transcription factors, NF-κB and AP-1 [412,454]. Activation of NF-κB by PCB 77 during the promotion of liver carcinogenesis was also noted [391]. The genotoxic effects of PCB 77 and PCB 77 in combination with PCB 52 have been reported [381,455,456]. PCBs that are AhR agonist (e.g. PCB 77, PCB 126) are categorized as hepatic tumor promoters in 2-stage hepatocarcinogenesis [357,380,384]. Recently PCB 77 was shown to be an initiator of rat liver carcinogenesis using a modified Solt Farber protocol [404].

PCB mixtures and congeners studied have generally diminished GPx1 activity in rat and mouse livers [317,406,411,419,446] although one study found no change in the GPx1 activity following Phenoclor DP6 treatment [369]. PCB 77 applied to Ah-responsive C57BL/6j and Ah-less responsive DBA/2N mice produced a lowering of GPX activity only in the Ah-responsive C57BL/6j wing mice at the maximal dose of 150 mg/kg suggesting that the Ah receptor may be involved in this effect [446]. Schramm et al. [411] also noted a depression in the GPx1 activities in the rat liver 2 weeks after PCB 77 and PCB 153 treatment. Likewise, a reduction in hepatic GPx1 activity was observed in both male and female rats treated with PCB 77 (100 μmole/kg administered twice per week) at 1, 2 and 3 weeks time points [317]. This study also found that the GPx1 mRNA levels were diminished in the same magnitude as GPx1 activity. They also reported that hepatic selenium decreased, which paralleled the changes in the GPx1 activity and transcript level.

In the present study, we found marked effects of PCB 77 on selenium levels and GPx activity in the treated female rats compared to the activity in male rats. The apparent sensitivity of female rats to PCB 77 effects is also seen in the increased liver weights, one of the prominent symptoms of PCB's toxicity. In Pelissier et al. [369], PCB mixture treatment (Phenoclor DP6) did not find a significant change in the hepatic GPx activity of

male Sprague-Dawley rats, although GPx activities in the two PCB treated groups were lower than the control groups. Our measurement of the hepatic selenium levels showed that the female rats have significantly reduced selenium levels at time points which correlates with the results shown for the glutathione peroxidase activity except for the 1 week time point where notwithstanding the variance, a decrease in selenium level is observable. PCB 77-treated male rats had no measurable difference in the selenium levels. In a previous study, PCB 77 reduced hepatic selenium levels and this reduction correlated with a decrease in the GPx activity levels as well as GPx1 transcript levels in both genders [317]. However, the effect on the female rats was also found to be more dramatic since activity went down to as low as 50% of the control. In addition, the GPx1 mRNA levels were more reduced for the female rats. Selenium levels were also markedly lower in the female compared with the male at all time points although no statistical comparison was made for the gender difference.

Based on these findings, there seems to be a gender difference in the effect of PCB 77 on hepatic GPx activity and selenium levels. It has been suggested that selenium metabolism in rats may be affected by gender because males may be storing more total body selenium and the selenium biological half-life is higher in males than females; and/or male rats have a higher selenium requirement than females [457]. It is interesting to note that indeed there is a gender difference as shown by the finding in mature Sprague-Dawley rats fed with 0.24 mg selenium per kg for 5 months, where males have lower Se concentration and GPx activity in the liver cytosols than the females [458]. This finding is mirrored in our results where the GPx activity and selenium level are higher for the female control and earlier time point groups compared with the male groups. Hepatic Se concentration was also found to be higher in female rats than male weanling rats fed with commercial rodent chow although no gender effect was found in the plasma or whole blood Se level and GPx activity [459]. In addition, no gender effect was seen in the Se concentration of muscle, brain, spleen and skin. Finley and Kincaid [458] also noted that in the kidney, the male rats had higher Se concentration and GPx activity than the females.

Assuming that under normal physiologic conditions, female rats tend to have higher hepatic selenium concentration and GPx activity, this does not explain the

apparent higher sensitivity of female rats to PCB 77 effects compared with male rats. It has been theorized that the sex difference in hepatic Se levels and GPx activity may be an effect of female hormones since it was shown that castration did not affect GPx activity in male rats whereas female rats given testosterone had lowered GPx activity [460]. Another example is the observation that Selenoprotein W levels were higher in the skin of females compared with male rats, suggesting that the control of transcriptional regulation of this selenoprotein may be affected by estrogen [459]. Hormonal control is also demonstrated by the selenium level in the testes [461].

Previous studies illustrated the effects of PCBs and TCDDs on selenium and we propose that PCBs may be affecting Se, and consequently or separately GPx activity, in the following ways:

1. PCBs potentiate selenium-deficient status by interfering with the utilization of dietary selenium by the body [408,462];
2. PCBs alters Se metabolism thereby affecting production of GPx [453]; and/or
3. PCBs alter the tissue distribution of selenium in different tissues by varying the demands on certain selenoproteins, which explains the lowered hepatic Se level [2,56,463].

PCB 77 administered to rats at gestational days 6 to 18 showed a reduction of serum testosterone due to PCB induction of P450 hydroxylases, which caused an increase in testosterone catabolism [464]. Rats exposed in-utero to TCDD, a xenobiotic that is similar structurally and functionally to PCB 77, showed reduced testosterone level in addition to decreased sperm counts [465]. Further, PCB 77 has been shown to change the potentials in electroretinograms in adult female but not male rats [466]. These observations together with the finding that female rats given testosterone had lowered GPx activity [460], suggest that PCB 77 has gender specificity and that selenium metabolism in the liver is altered by modulating sex-steroid dependent functions. When studying coplanar PCBs toxic effects, it is necessary to consider gender effect in experimental designs. Likewise, study of selenium and selenoproteins in relation to PCBs or other environmental contaminants should take into account the gender status of exposed animals.

Notwithstanding the gender effect, the fact remains that PCB 77 appears to cause a reduction of Se levels in the liver. We hypothesize that PCB 77 influences the mobilization of Se by: 1) increasing the excretion rate via increased methylation process or by limiting the metabolism of Se in the blood into forms that are utilizable by the liver; 2) reducing the liver uptake of the selenium forms from the blood; or 3) increasing the synthesis of more crucial or Se-deficiency-sensitive selenoproteins (selenoproteins P and W) that are then exported to the muscles or blood. Selenoprotein W is found mostly in muscles while selenoprotein P is found in high levels in the plasma compared with the liver and other organs [55,94,467-469]. Interestingly, PCB 77 was shown to have a relatively high affinity for blood [470]. In addition, compared with other PCB congeners, PCB 77 did not have a very high affinity for adipose tissue. Since PCB 77 appears to bind to blood, possibly to blood lipoproteins, it is conceivable that PCB 77 may interfere with the transport of Se from the circulation into the liver.

In conclusion, our studies show that a co-planar PCB may significantly alter the redox status of the liver by reducing the anti-oxidant activity of glutathione peroxidase. In addition, the effect of PCB 77 on the hepatic selenium level and GPx activity is possibly influenced by gender. Considering the questions posed by the results of this study and with the increasing interest in using selenium in cancer or tumor prevention together with other vitamin anti-oxidants, it is important to understand the effects of coplanar PCBs and likewise TCDD on selenium metabolism and utilization and on the synthesis of selenoproteins that provide protection against oxidative damage.

CHAPTER 4: EFFECTS OF 3,3',4,4'-TETRACHLOROBIPHENYL (PCB-77) ON THE DISTRIBUTION AND METABOLISM OF SELENIUM IN RATS

Abstract

Se (Se) is an essential element that may influence the development of cardiovascular diseases and cancer. Environmental contaminants, like polychlorinated biphenyls (PCBs), produce oxidative stress, alter Se distribution/utilization, and affect antioxidant systems, including glutathione peroxidase, a selenoprotein. 3,3',4,4'-tetrachlorobiphenyl (PCB-77) is a dioxin-like, coplanar PCB that has been shown to reduce glutathione peroxidase activity in the liver. The aim of this study was to determine the effect of PCB-77 on the metabolism and distribution of Se. Following PCB-77 i.p. bolus dose administration (300 μmol/kg), we examined the hepatic glutathione peroxidase and thioredoxin reductase activities, the daily excretion, and the 21-day post treatment tissue distribution of Se in Sprague-Dawley rats. The Se level in the liver after PCB-77 treatment was repressed and was accompanied by a decrease in the hepatic glutathione peroxidase activity. In contrast, thioredoxin reductase activity was not affected by PCB-77. The Se levels concentration in extrahepatic organs remained unaffected; however, the relative Se content (% of control) was increased in the spleen and muscle. In addition, PCB-77 affects urinary Se excretion patterns whereas fecal Se excretion was not altered.

Introduction

Selenium (Se), an essential micronutrient, has been well studied for its anti-cancer activities; however, the mechanisms for its chemopreventive action remains to be clearly elucidated [117-119,184,185,188,471-473]. Although the best understood function of Se is the antioxidant properties of Se-containing enzymes, like glutathione peroxidase (GPx) and thioredoxin reductase (TrxR), it is now known that Se may have more complex roles in cancer, cardiovascular and immune disorders [197,471,472,474,475].

Currently there are 25 genes known to encode selenoproteins in the human genome, although not all have known functions [46]. Of these, GPx, TrxR and thyroid hormone deiodinases (DI) are well characterized. Selenoprotein P (SePP), which is mainly found in plasma and reported to be an antioxidant, has been shown to function as a Se-supply protein by transporting hepatic Se to other tissues [55,100,476]. Bioavailability of Se from the diet depends on its biochemical nature. Understanding Se metabolism is a key consideration in determining its efficacy as a chemopreventive agent [477]. Se metabolism involves the transport of absorbed dietary Se via the blood to tissues mainly liver, conversion of inorganic and organic Se to selenide, Se-containing protein synthesis, and methylation [56,117,136,173,193,478-480]. The methylation reaction involves the production of monomethylated Se or methylselenol which many believe maybe the Se metabolite responsible for the chemopreventive effect of Se [117,120,472, Ip 2002].

Polychlorinated biphenyls (PCBs) a family of chemicals comprised of 209 compounds were widely used because their stability under chemical, physical and electrical conditions made them suitable for various industrial applications. Although production has ceased, the lipophilicity and chemical resistance allowed PCBs to be bioconcentrated, bioaccumulated and biomagnified leading to their persistence and global transport as organic pollutants [251,255]. Studies on exposed human population and animals have provided evidence that PCBs contribute to cancer as well as cardiovascular diseases [258,315,357,481-485]. In some findings, gender specificity was found to contribute to PCBs induction of cancer [315,486].

One of the mechanisms implicated for PCBs induced tumorigenesis is oxidative stress [255,258,487,488]. PCBs increased oxidative stress in cells and tissues by

enhanced lipid peroxidation and the induction of cytochrome P-450 that produce reactive oxygen species (ROS) as a by-product [489,490]. In addition, metabolism of PCBs into catechols and hydroquinones contribute to oxidative stress when these compounds are oxidized to semiquinones and quinones, which produce ROS [491]. Moreover, oxidative damage to cells and tissues by PCBs may be exacerbated through depletion of Se and depression of Se-dependent GPx (GPx1) activity [317,408,411,446,462,492]. However, there is little knowledge on how PCBs may be affecting Se metabolism.

In this study we determined the effect of PCB-77 on the metabolism and distribution of Se. We proposed that reduced glutathione peroxidase activity results from hepatic depletion of Se may be a consequence of PCB-77 enhanced transport of Se to other tissues as well as increased Se excretion. To follow the fate of Se, we conducted a metabolic study using female Sprague-Dawley rats administered with a single dose of PCB-77 (300 µmol/kg).

Materials and Methods

Chemicals

PCB-77 (3,3',4,4'-tetrachlorobiphenyl) was synthesized, purified and characterized as described [411]. All other chemicals were purchased from Sigma Chemical Co. unless indicated.

Experimental design

Female Sprague-Dawley rats (150-174 grams) were housed individually in hanging, wire mesh metabolic cages. The Institutional Animal Care and Use Committee of the University of Kentucky approved the animal protocol. Rats were provided pulverized form of commercial unrefined rat chow, which is reported to contain approximately 0.2 mg Se/kg diet (Harlan Teklad, St. Louis, MO) and water ad libitum. The animals were acclimatized in the metabolic cages for one week before starting the study. A single i.p dose of PCB-77 dissolved in corn oil at a dose of 300 μmol /kg body weight was administered to 4 rats while the other four received the corn oil vehicle. Body weights and food consumption were recorded every 2 days during the experiment. Water consumption was recorded during the latter 10 days of the study when large differences in the urinary output of the PCB-77 treated rats were noted. Urine and feces, collected daily in the morning, were measured, aliquoted and stored at −80°C. The sample collection component of the cages was washed daily to reduce contamination of the fecal and urine samples by food debris. The rats were sacrificed 21 days after PCB and corn oil treatment. Rats were euthanized, and liver, heart, kidney, lung, brain, thymus, spleen, muscle, and intestine obtained and processed for later analysis were stored at −80°C.

Sample preparation for Se determination

Approximately 0.2 to 0.5 gram wet tissue was weighed and placed in a microwave digestion vessel. Four ml of urine were freeze-dried first, and then reconstituted in 0.5 ml deionized water. Feces were freeze-dried, ground, and ≈ 0.7-1.0 gram dry weight was weighed into digestion vessels. Two ml pure grade concentrated nitric acid was added to the samples in the vessels; for fecal samples, 4 ml nitric acid was used. The samples were digested using a computer controlled microwave digester (CEM

MSP 1000) employing different programs for each sample matrix (Table 4.1). At the end of the digestion the acid digest in the vessels were cooled, transferred into polyethylene centrifuge tubes and immediately diluted with deionized water to make up a 6-10 ml volume.

Table 4.1: Digestion program using CEM 550 Microwave Digester

Sample Matrix	Stages:	1	2	3	4
Tissues	Pressure (psi)	050	100	150	
	Time (min)	10	10	10	
Urine	Pressure (psi)	040	080	120	
	Time (min)	8	8	14	
Feces	Pressure (psi)	040	080	120	160
	Time (min)	8	8	20	20

Determination of total Se

Se was measured by a graphite furnace atomic absorption spectrophotometer (GFAAS) equipped with a Zeeman background correction factor and an autosampler (Varian-Tech, USA). The GFAAS default program set by the manufacturer was used with some modifications (Table 4.2). A 500 mg/l palladium (Pd) modifier in 1% citric acid was used to prevent the sample from evaporating during the ashing stage. A blank and three Se standards were set up using a 100 µg/l bulk solution prepared from an AAS Se standard solution. A 5 or 10 µg/l reference Se solution (SPEX, USA) was used as internal standard.

Table 4.2: GFAAS Se analysis program

Program Parameters

Element - Matrix: Se
Instrument Type: Zeeman
Instrument Mode: Absorbance
Calibration Mode: Concentration
Measurement Mode: Peak Height
Replicate standard and samples: 3
Wavelength: 196.0 nm
Slit Width: 1.0 nm
EHT: 289 Volts
Lamp Current: 15.0 mA
Background Correction: BC On
STANDARD 1:   5.000 ug/L
STANDARD 2:   10.000 ug/L
STANDARD 3:   30.000 ug/L
Reslope Lower and Upper Limit: 75.0 %, 125.0 %
Recalibration Rate: 10
Calibration Algorithm: Linear Origin
Cal. Lower and Upper Limit: 20.0 %, 150.0 %
Total Volume: 25 uL
Sample Volume: 20 uL
Modifier: 500 ppb Palladium in 1 % Citric acid
Modifier 1 Vol.:   5 uL
Rinse solution: 1% HNO_3 in 0.1 % Triton X-100
                                and 0.01 % Antifoam B

Stages	Step	Temp (C)	Time[493]	Flow (L/min)
Drying	1	85	5.0	3.0
Drying	2	95	50.0	3.0
Drying	3	120	12.0	3.0
Ashing	4	1300	11.0	3.0
Ashing	5	1300	10.0	3.0
Ashing	6	1300	2.0	0.0
Atomization	7	2600	0.8	0.0
Atomization	8	2600	2.0	0.0
Cleaning	9	2800	2.0	3.0

Limitation of the total Se analytical method

In a subsequent study (Chapter 6), the microwave digestion and Zeeman-GFAAS method of selenium analysis was evaluated against two other methods, the Neutron Activation Analysis (NAA) and fluorometric analysis methods. This method comparison study found that the selenium concentration in the urine, fecal and liver tissue samples using the Zeeman-GFAAS method was lower than that resulting from the NAA and fluorometry-analyzed samples. The reader or user of this paper is therefore cautioned that the selenium concentration shown in the results of this study is lower than the expected value. However, since all the urine, fecal and liver tissue samples of both the control and the PCB 77 treated rats were analyzed using the same method, we assumed that the comparison between these two groups are still valid.

Protein assay

Protein concentration of supernatants, dialysates and cytosolic fractions was determined using the BCA method (Pierce Chemical Company). Briefly, 200 μl of mixed reagent, 1 part Reagent A (Cu^{+1}) and 50 parts Reagent B (bicinchonic acid), was added to each well of a 96-well plate. 10 μl of each diluted sample or bovine gamma globulin standard (0. 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml) was added to each well in duplicate. After incubating the plate for 30 minutes at 37°C, the plate was cooled to room temperature and read at 562 nm with a Bio-Tek plate reader. Using the KinetiCalc software, the concentration of protein (mg/ml) was calculated for each sample based on the standard curve.

Liver homogenates and cytosolic fractions preparations

Approximately 0.5 gram liver was used in the preparation of liver homogenate as previously described [447]. Briefly, the liver was homogenized in 0.25M sucrose/0.1mmol ethylenediaminetetraacetic acid (EDTA), pH 7.4 using an Ultra-Turrax homogenizer, then centrifuged at 10,000xg for 20 minutes. The supernatant was collected and then centrifuged at 100,000xg for one hour. After separating the cytosolic fraction (supernatant) from the microsomal pellet, the cytosolic fraction was aliquoted for protein determination and enzyme assay.

Glutathione peroxidase activity assay

The glutathione peroxidase activity of the cytosolic fraction was determined using the method of Paglia and Valentine as modified by St. Clair and Chow [199,449]. Briefly, a reaction mixture of 2 mmol EDTA , 2.5M sodium azide (NaN_3), 0.2 mM NADPH, 1 mM GSH, 1 unit/ml glutathione reductase in 50mM Tris-HCl (pH 7.6) was prepared. In a quartz cuvette, 10 µl of cytosolic fraction diluted with Tris-HCl ($\approx$10-20 µg protein) and 940 µl reaction mixture was pre-incubated for 1 min at room temperature. 30% H_2O_2 was added into the reaction cuvette and mixed. The decrease in absorption was monitored spectrophotometrically for 60 seconds (10 readings at 6 seconds interval) at 340 nm. For total glutathione peroxidase activity, 25 µl cumene hydroperoxide (1.2 mM) was rapidly added and absorbance was monitored for 1 minute at 340 nm. The enzyme activity was expressed as nmoles NADPH/min/mg protein.

Homogenate and dialysate preparation

Frozen liver tissues (0.45-5.0 g) were homogenized in phosphate buffered saline (PBS) pH 7.4 with 1 mM EDTA solution for 30 seconds using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 1300 g for 30 minutes and the supernatant collected for dialysis. The protein concentration of each supernatant was adjusted to 8 mg/ml protein with the PBS-EDTA solution prior to dialysis. The supernatant was dialyzed in PBS pH 7.4 to remove endogenous GSH. Approximately 1 ml of supernatant was pipetted into prepared dialysis tubing, clipped to close, and placed into a beaker containing PBS solution (100 ml PBS/1 ml supernatant) for 16 hours at 4°C. The buffer was replaced at least one time. The dialysate was collected and aliquoted. The protein concentration of the dialysate was adjusted to 1 mg protein/ml [11].

Thioredoxin reductase (TxrR) activity assay

The TrxR activity was determined using a method of Holmgren and Bjornsted [494] as modified by Hill et al. [11]. Briefly, a reaction mixture was prepared containing 1 ml of 10 mg/ml insulin, 400 µl of 1M HEPES buffer (pH 7.6), 80 µl of 0.2M EDTA and 80 µl of 40mg/ml freshly prepared NADPH. 60 µl of the reaction mixture was added into tubes. Two tubes were allocated to each dialysate sample. To one tube, 15 µl of 60

μM E. coli thioredoxin (test) was added while 15 μl of distilled water was added to the other tube (control) to represent the non-TR-thioredoxin system-dependent reaction. After adding 105 μl (≈105 μg protein) of dialysates into the test and control tubes, the reaction was incubated at 37°C for 15 minutes. The reaction was stopped by adding 750 μl of 0.4 mg/ml DTNB (5,5'-dithiobis(2-nitrobenzoic acid) in 6M guanidinium hydrochloride and the absorbance of both test and control mixtures were measured at 412 nM. The TR-thioredoxin system-dependent NADPH reduction of insulin was determined by subtracting the absorbance of the control from that of the test reaction mixture. TrxR activity was expressed as A_{412} units x 1000 / (min x mg protein).

Statistical analysis

Data are presented as the mean ± SEM. The difference between the treated and control groups were examined using a student t-test. A value of $P < 0.05$ was considered statistically significant.

Results

Body Weight Changes

Day 0 corresponded to the body weights measured prior to administering PCB-77 or corn oil to the rats. Overall, PCB-77 had no effect on body weight changes compared with the control (Figure 4.1). Although continuous increase in weight from day 0 to day 19 was observed for the PCB-77 group, the weight gain compared with the control started to decrease at day 3. Consequently, the body weights were consistently lower than the control from day 3 up to the end of the study although the differences were not significant.

Food and Water Consumption

The amount of feed consumed was examined to determine any PCB-77 related effect on food intake resulting in weight loss or decreased weight gain. The initial food intake was the same for both groups until day 3 when the PCB-77 treated rats had a significant drop in food consumption. The significantly reduced food intake continued until day 7 after which food consumption increased and became similar to the control. The decreased food intake would explain the observed lower body weights of the PCB-77 group.

Water intake was monitored when we noticed a large difference in urine output between individual rats in the PCB-77 group. We found that although the variance in urinary output between the PCB-77 treated animals was large, the mean water intake was not significantly different between the groups (data not shown).

Fecal Output and Se in Feces

Throughout the observation period, although the PCB77-treated rats had more fecal output, the mean excretion weights were not significantly different compared to the control group (Figure 4.3a). The fecal output of the PCB-77 group showed peaks at day 15 and day 19. PCB-77 did not affect the total Se excreted through the feces all throughout the observation period (Figure 4.3b). Interestingly, Se excretion peaked at day 15 for both groups but not on day 19. On both days the fecal output was at its highest.

Urinary Output and Se in Urine

PCB-77 did not have an effect on the urinary output compared with the control (Figure 4.4a). The large variance around the mean of the PCB-77 exposed group was a result of the large difference in urine output of individual rats in the PCB-77, which ranged from 5 to 26 ml whereas the urinary daily output of the control group ranged from 10 to 19 ml.

The Se excreted in urine at day 3 to day 7 was significantly decreased in the PCB-77 treated rats compared to the control, after which it increased to the same level as the control (Figure 4.4b). The decrease in the level of Se in urine correlated with the decreased food consumption by the PCB-77 rats (Figure 4.2). Interestingly, the amount of Se excreted through urine increased significantly at day 17 and 19 compared to the control. This time, the increase was not a result of a corresponding increase in food consumption. The urinary Se level of the PCB-77 treated group dropped at day 21, which may again be due to a drop in the food consumption.

Se Levels in Tissues and Organs

The Se concentration in different tissues was examined. Figure 4.5 shows that PCB-77 significantly decreased the liver Se concentration only. Although Se concentration in the heart, lung, spleen, adrenal, thymus and muscle appeared to be increased, the difference was not significant.

Looking at the total Se content in each organ, we observed that the PCB-77 treated rats had Se content in the whole liver reduced by almost 13 % compared to the control (Figure 4.6). Thymus Se was also decreased by approximately 11% compared to the control. On the other hand, the Se content of the spleen and muscle was increased by 41% and 28%, respectively. The total Se in muscle was calculated by multiplying the concentration by 45% of the rat body weight [495].

Se Distribution and Excretion in Relation to Se Dietary Intake

Table 4.3 gave an overall picture of the relative distribution and excretion of selenium expressed as % of the total dietary Se. Compared to the control rats, the total amount of Se in the rat chow consumed by the PCB-77 treated rats during the duration of

the study (22 days) was not significantly different (data not shown). The proportion of total Se excreted in the urine throughout the 22 day observation period in relation to Se dietary intake was almost similar for both the control and the PCB 77 groups whereas a slight decrease in the PCB 77 group was observed for the fecal Se (Table 4.3). The proportion of hepatic Se level of the PCB treated group was decreased compared to the control. The opposite was observed for the combined Se for analyzed organs (kidney, heart, lung, spleen, adrenal, brain, thymus and muscle) excluding liver (Table 4.3 and Figure 4.6). A slight decrease in the proportion of selenium intake of the PCB 77 treated group was observed. This could be explained by the decreased food consumption from Day 3 to Day 7 (Figure 4.2). The Se in the feces and liver of the PCB 77 treated rats was 95% and 87% of the control group, respectively. In contrast, the proportion of Se in PCB77-treated organs exclusive of the liver was increased in proportion to the control group.

Glutathione Peroxidase and Thioredoxin Reductase Activities

PCB-77 significantly decreased both hepatic Se and Se-dependent glutathione peroxidase activity (Table 4.3). The total glutathione peroxidase activity was also significantly decreased as a result of PCB-77 treatment. In contrast, PCB-77 treatment had no significant effect on the hepatic thioredoxin reductase activity.
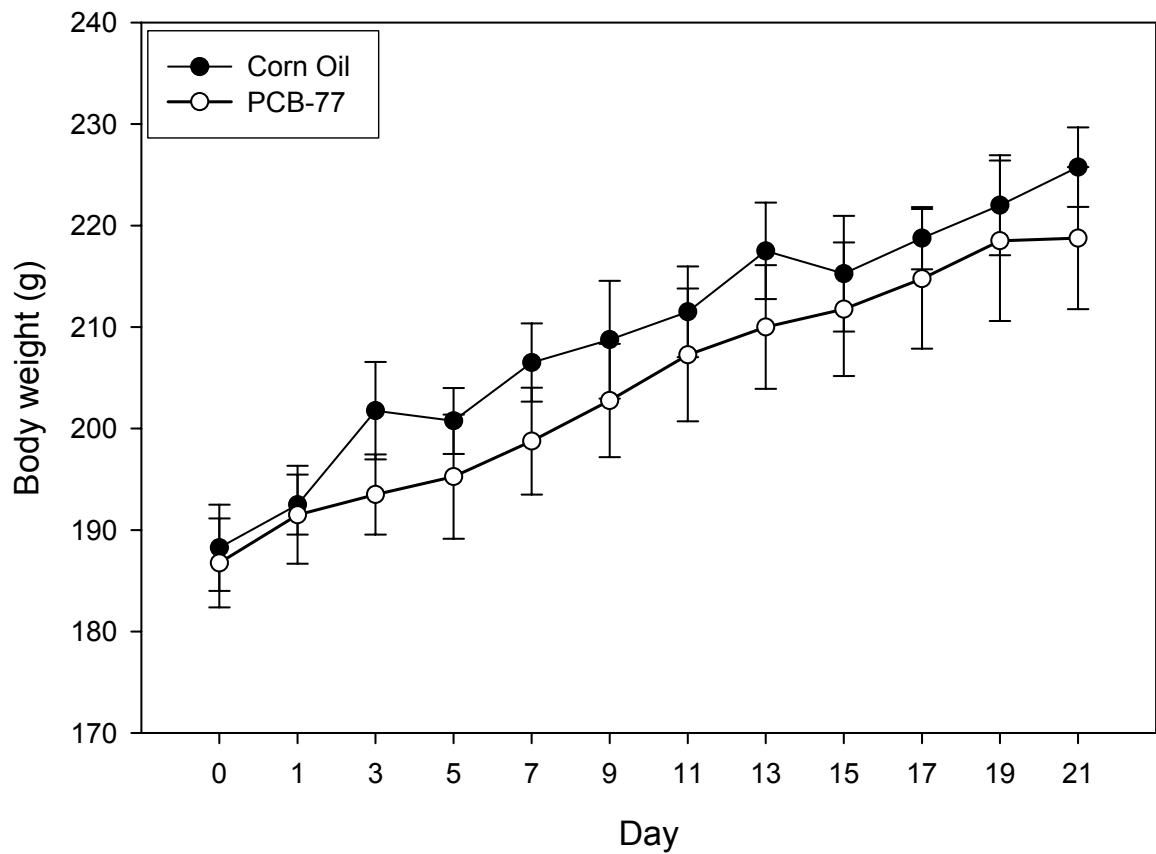
Figure 4.1: Body weight of female rats that received a bolus dose of PCB 77 (300 µmol/kg) or corn oil. Each point is the mean ± SEM of four rats.
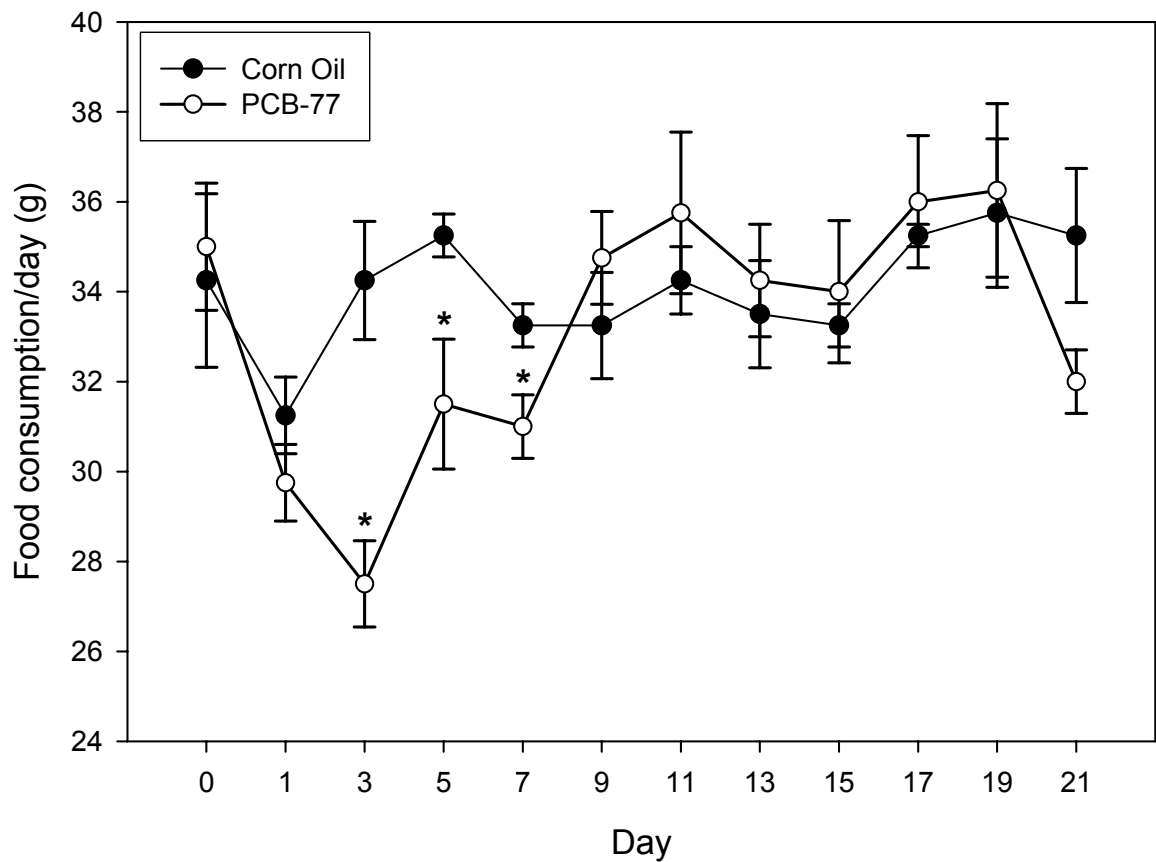
Figure 4.2: Food consumption of female rats that received a bolus dose of 300 µmol/kg PCB-77 or corn oil. Each point is the mean ± SEM of four rats. *P < 0.05 compared with control.
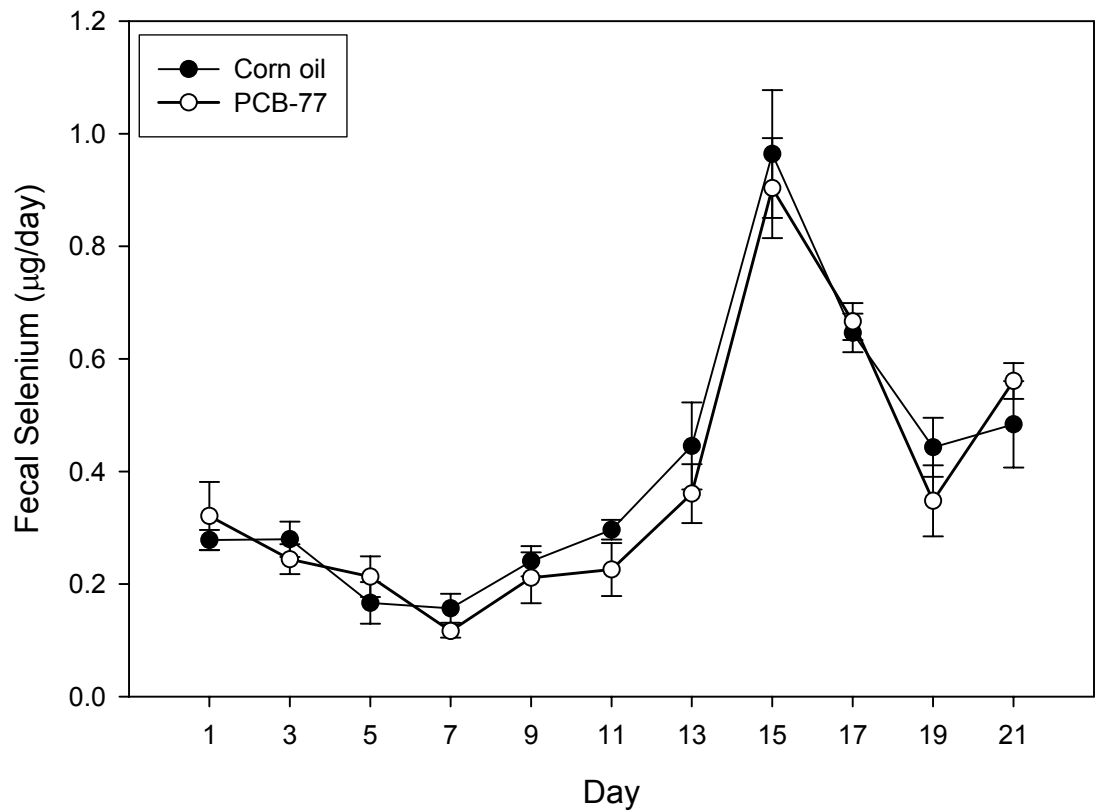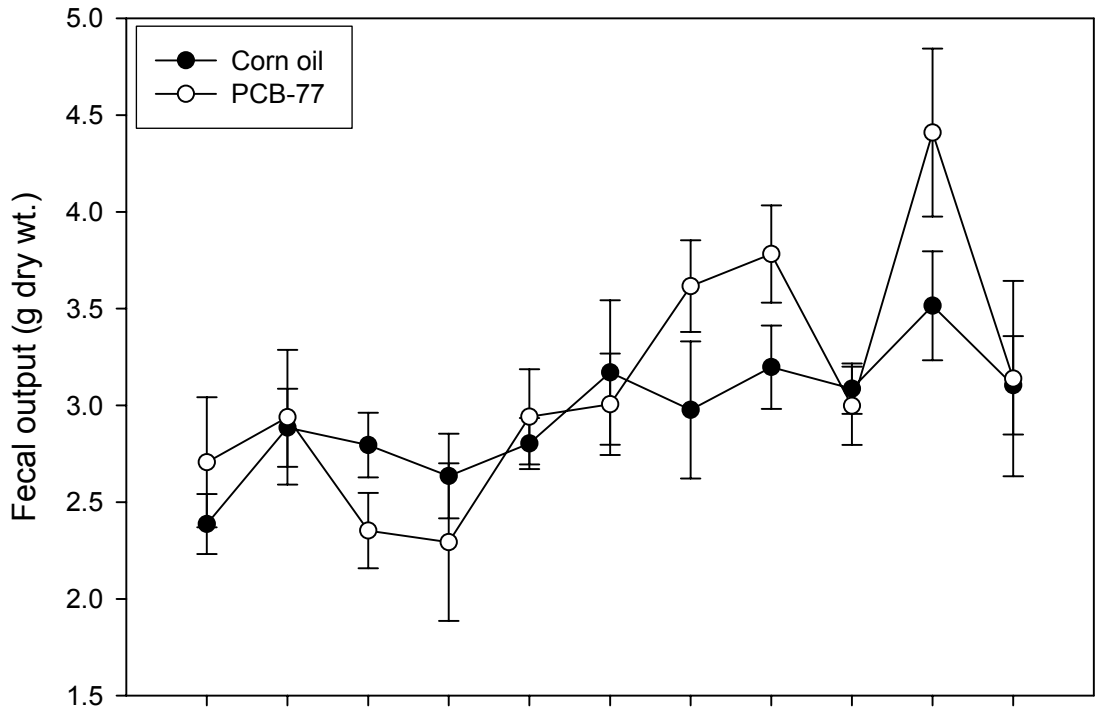
Figure 4.3a and b: Fecal output per day and fecal Se expressed as µg Se/day. Female Sprague Dawley rats received a bolus dose of 300 µmol/kg PCB-77 or corn oil. Each point is the mean ± SEM of four rats. Selenium levels were measured using the Zeeman-GFAAS method.
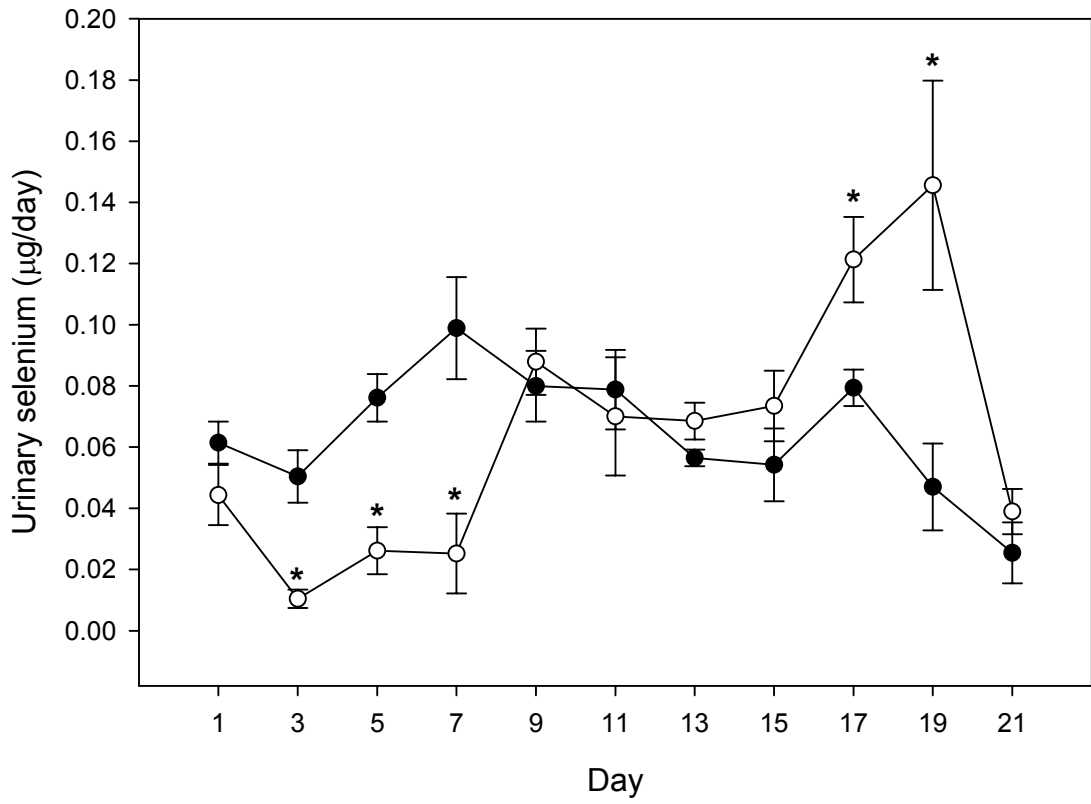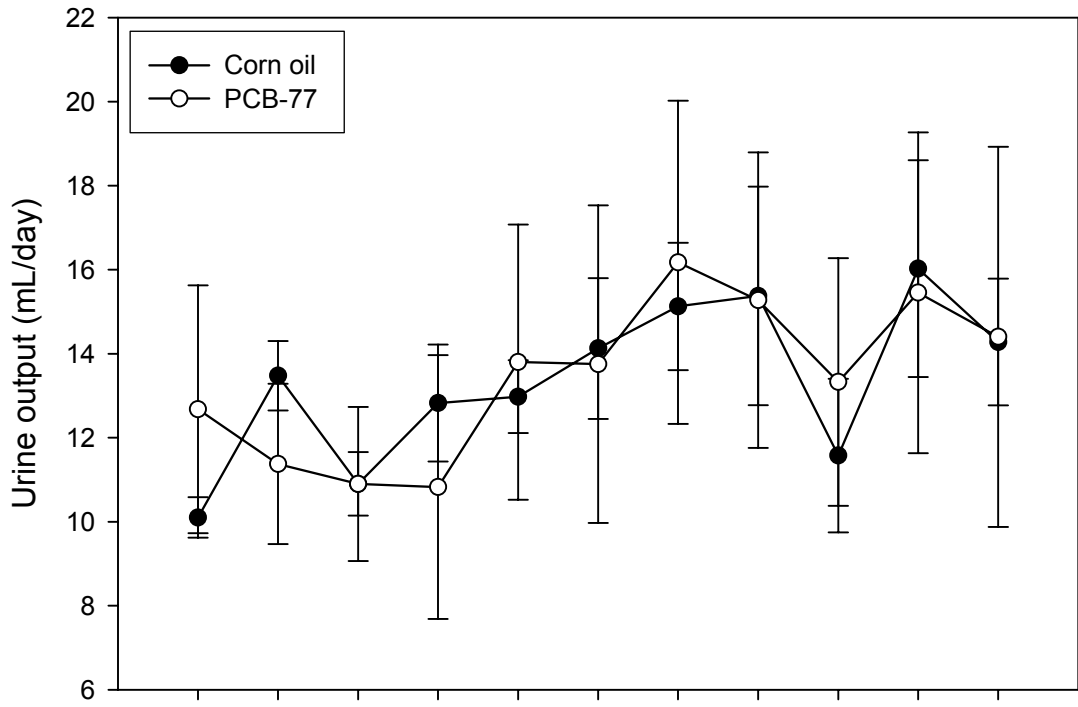
Figure 4.4a and b. Urinary output and urinary Se excreted per day. Female Sprague Dawley rats received a bolus dose of 300 μmol/kg PCB-77 or corn oil. Each point is the mean ± SEM of four rats. Selenium levels were measured using the Zeeman-GFAAS method.
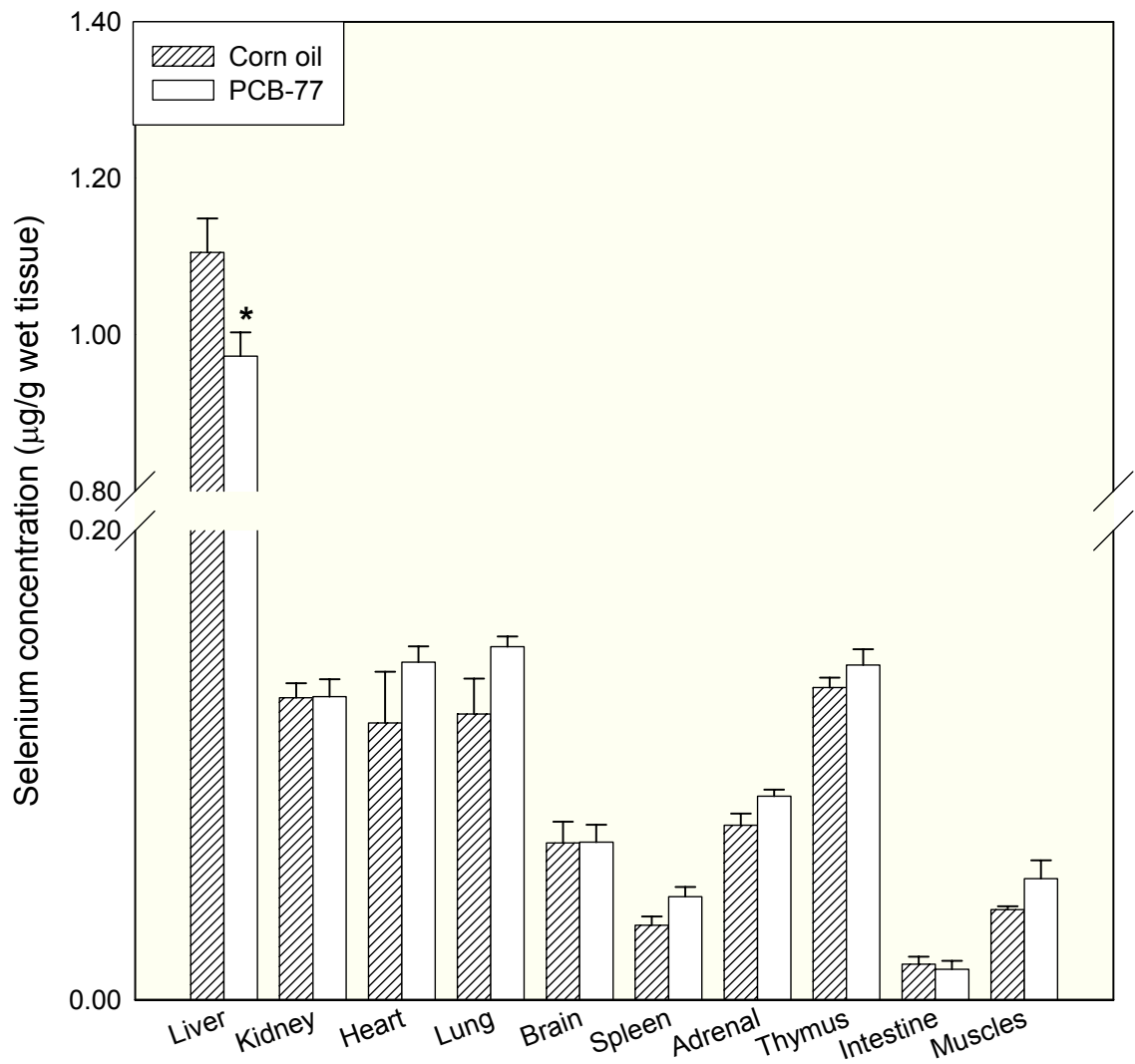
Figure 4.5: Se concentration in various organ tissues of female rats that received a bolus dose of 300 µmol/kg PCB-77 or corn oil. Each point is the mean ± SEM of four rats. *P < 0.05 compared with control. Selenium levels were measured using the Zeeman-GFAAS method.
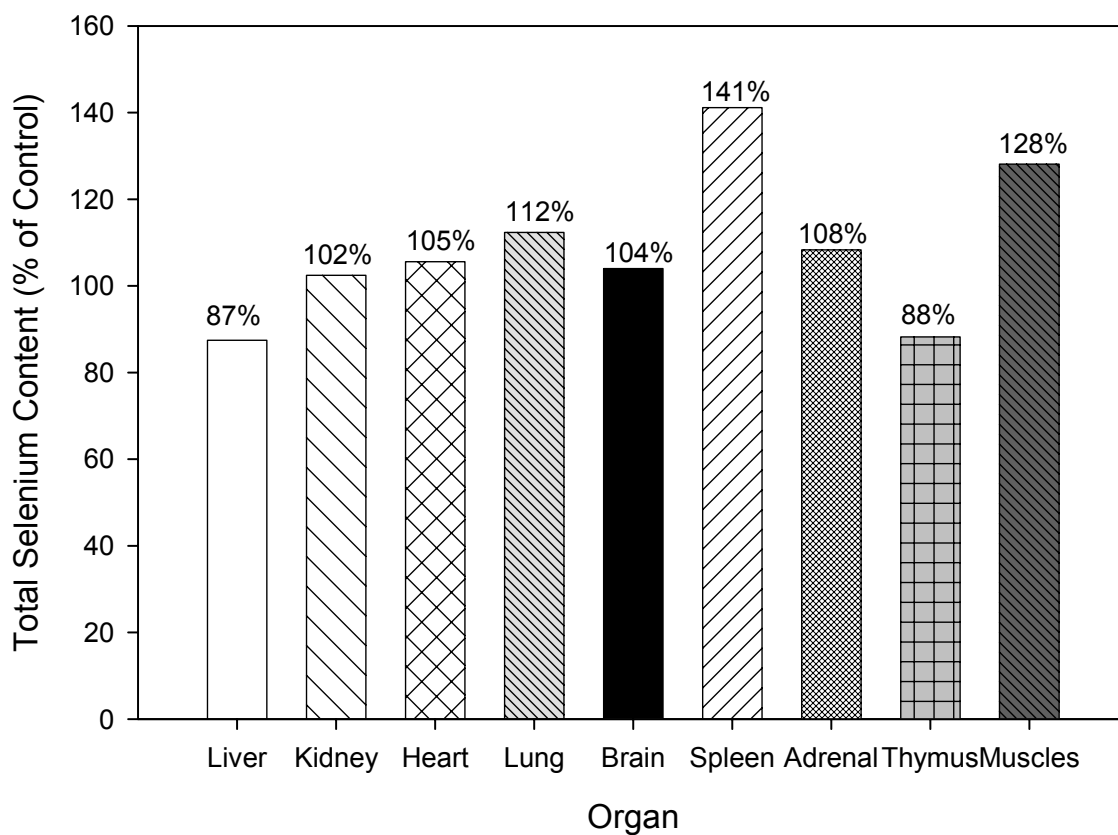
Figure 4.6: Relative Se content of organs of PCB 77-treated female rats (mean Se level of PCB-77 group / mean Se level of control group x 100). Selenium levels were measured using the Zeeman-GFAAS method.

Table 4.3: Overall distribution of Se in relation to Se dietary intake

	Control	PCB 77	
	% of dietary Se[†]	% of dietary Se[†]	% of Control[*]
Se from Diet			99
Se in Urine	3.47	3.53	101
Se in Feces	21.55	20.69	95
Se in Liver	13.36	11.83	87
Se in Other Organs excluding Liver	11.21	14.19	125

[†]The proportion of the 22 day total Se in the urine, feces and organs in relation to the total Se dietary intake expressed in percent. The mean was calculated from 4 rats per group. [*]The ratio of the calculated 22 day-total Se in the diet, urine, and feces and Se levels in organs of the PCB-77 rats to the control group Se values, expressed in %.

Table 4.4: Glutathione peroxidase (GPx1) activities, thioredoxin reductase (TrxR) activity, and Se concentration†

	Corn Oil	PCB-77
GPx1	1690 ± 30.8	1344 ± 66.2*
Total GPx	1843 ± 73.6	1553 ± 24.1*
TrxR	31.2 ± 2.1	36.6 ± 3.1
Se	1105 ± 43.3	972 ± 30.6*

†Values are mean ± SEM of four rats. GPx1 and total GPx activities are expressed as nM NADPH/min/mg protein. TrxR is expressed as A_{412} units x 1000/ (min x mg protein). *P < 0.05 compared with the control.

Discussion

The PCB-77 congener is a potent AhR agonist, an efficacious inducer of CYP-450 1A and has a high toxic potency [251,255,335]. Our group previously showed that PCB-77 treatment decreased hepatic GPx1 activity, which correlated with a decrease in GPx1 mRNA [317]. In this study, we followed the excretion of Se in feces and urine as well as the distribution of Se in tissues after administering a bolus dose of PCB-77. We found that the urinary excretion of Se at 17 and 19 days after exposure to PCB-77 was significantly increased in rats despite the lack of change in their urinary output compared with the control rats. In addition, PCB 77 administration resulted in decreased hepatic selenium and GPx1 activity whereas TrxR activity was unaffected. Except for liver, the Se concentration in other organs were not significantly altered; however, the relative Se content of the PCB treated rats (% of control level) was increased in the spleen (141%) and in muscle (128%).

Se in the diet is readily absorbed and processed mainly in the liver into selenoproteins where inorganic Se is reduced by glutathione via selenodiglutathione into hydrogen selenide [94,100,141]. Selenomethionine and selenocysteine, the major dietary Se forms found in plants are converted into hydrogen selenides by B-lyase [30,32,117]. The selenides are detoxified or transformed into methylated metabolites by a methyltransferase enzyme in the presence of S-adenosyl methionine [137,143,496-499]. Although Se metabolism and distribution in different tissues, and selenoproteins synthesis, expression and activities have been largely elucidated, Se transport and excretion pathways remain unclear. It is known that sequential methylation of selenides produce methylated Se metabolites, that are excreted either in urine or breath and the liver appears to be the main organ for Se methylation although it was reported that methylation also occurs in the kidney [116,136,139,141,143,498,500-502]. Urine is the main excretion route for Se and methylated metabolites and serves to maintain Se homeostasis [136]. Se is eliminated in breath as dimethyl selenide only when Se dose is high or excessive. Our results showed that PCB-77 affects Se excretion in urine, but not feces. We found that the urine output 24 hours after PCB-77 treatment did not differ from the control rats. Likewise, no concomitant change in urinary Se was observed at 24 hours.

In contrast, urinary Se was significantly lowered on days 3, 5 and 7 after PCB-77 dosing. Since food consumption of the PCB-77 group was significantly decreased starting Day 1 to Day 7, we concluded that the reduction in urinary Se may be a consequence of reduced dietary intake, an indirect PCB 77 effect. Similar to our results, a study found that male rats treated with cyanide and then injected with ^{75}Se-selenite had increased Se excretion in urine but not in feces; however no change in radioactivity in liver, kidney, muscle or testes was observed [503]. A second experiment using cyanide only demonstrated that glutathione peroxidase and Se concentration was decreased in the liver, kidney, muscle. In our study, glutathione peroxidase activity and Se was also decreased in the liver of female rats given PCB 77; however, Se concentration of kidney and muscles was not affected.

Metabolites in urine of rats fed with adequate dietary Se (0.2 mg/kg) were observed to consist mostly of monomethylselenol (MMSe, CH_3SeH) followed by trimethylselenonium and inorganic Se [141,504]. At higher dietary Se, the excretion of the last two Se forms was increased and this was accompanied by increase in total urinary Se. Since PCB 77 increased urinary excretion of Se at adequate Se intake, it is possible that PCB 77 may be affecting the methylation process and therefore disturbing Se homeostasis. It was observed that when Se methylation reaction is depressed, Se concentration in urine of mice was high, which indicates that the inorganic Se in urine may be the form contributing to increased Se excretion [143]. Recently, another major Se metabolite in the form of selenosugar was observed in rats and human urine. This selenosugar was also detected in the liver; however its role in Se metabolism is unknown [505].

Combs and Scott found that PCBs depressed glutathione peroxidase activity and indicated that the ability of PCBs to induce the cytochrome P-450 may lead to a decrease in the biological utilization [408]. They proposed that this effect may be due to a change in the oxidation state of Se, which may result in the preferential incorporation of Se into compounds that are not "biologically useful". PCB 126, another coplanar PCB and potent AhR agonist was shown to induce a 54-kDa Se binding protein in rat liver that is similar to a nuclear 56 kDa Se- binding protein [506-508]. The induction of this protein appears

to be associated with the AhR; however, the function of this protein is not known and may possibly be a selenium containing protein that is not biologically useful.

Evidence that an AhR-linked mechanism may be involved in the ability of PCBs to reduce GPx1 activity was shown when Ah-responsive C57BL strain of mice produced a significant decrease in GPx1 activity after PCB 126 treatment, whereas no effect was observed in the Ah-less responsive DBA strain of mice [413]. However, this premise was faulted with the finding that the battery of genes that are linked to the xenobiotic response element (XRE) did not include the GPx gene [509,510]

Thioredoxin reductase (TrxR) is a selenocysteine containing flavoprotein that catalyzes the reduction of thioredoxin. Among other functions, the thioredoxin system is important in embryonic development and was found to be over-expressed in human primary tumors [210,511,512]. Our group has shown that PCB-77 strongly promotes hepatic tumor in rodents through different mechanisms including oxidative stress, increased cell proliferation rate and induction of NF-kB and AP-1 [357,391,513]. PCB-77 did not affect the thioredoxin reductase activity unlike its effect on GPx1 and total GPx. This observation mirrors the differential response of selenoproteins to Se status. During Se deficiency, GPx1 activity is known to decrease dramatically [514] whereas TrxR activity is not as adversely affected [11,515,516]. Moreover, the effect on the activity of selenoproteins does not correspond to a similar change in the mRNA levels [515] Although Se deficiency produces a TrxR loss of activity, the mRNA level is conserved indicating that this selenoprotein is more important to cell functioning than GPx1. Our results showed that the TrxR activity of the PCB-77 exposed animals is conserved compared to GPX1. Thioredoxin and TrxR were found to be induced in malignancy systems while GPx1 is depressed [204,517]; hence, since TrxR remained unaffected by PCB-77 imply that the thioredoxin system through TrxR may be incorporating hepatic selenium at the expense of GPx1.

Another possible mechanism for PCB-77-induced depletion of hepatic Se may be through selenoprotein P (SePP). SePP is a plasma selenoprotein that contains up to ten Sec residues per molecule. The liver is the primary source of plasma selenoprotein P (SePP), which binds 60-70% of the Se in plasma [95]. Studies including knock-out models for SePP have shown that this plasma selenoprotein plays a role in Se transport

and tissue distribution [94]. Changes in the synthesis and secretion of SePP were found to affect the Se level and the expression of GPx and TrxR in blood and tissues [55,101-103]. Although the Se concentrations in tissues were not altered by PCB-77, when we examined the relative total Se content of tissues, the spleen and muscles of PCB 77 rats had increased total Se content compared with the liver. In addition, PCB 77 decreased the relative distribution of Se in the liver and the feces, and the opposite was observed for the combined selenium of all analyzed organs excluding the liver. PCB 77 and its metabolites were reported to bind covalently to rat hemoglobin and have a relatively high affinity for erythrocytes [352]. These observations point to the possibility that PCB 77 may be interfering with Se distribution through mechanisms involving the interaction between hemoglobin bound PCB 77 or its metabolites and SePP. The increased proportion of combined Se in the extra-hepatic organs presumably due to the increased Se level in the spleen and muscles support the hypothesis that PCB 77 may be inducing extra-hepatic transport of Se via SePP. Further studies on the effect of PCB-77 on the distribution of GPx1, TrxR, SePP and extracellular GPx (eGPx) in extra-hepatic organs could elucidate the extra-hepatic tissue transport hypothesis.

In summary, PCB-77 decreased the Se content of the liver and depressed GPx1 activity. These PCB-77 effects may be a result of increased excretion of Se in urine, but not in feces. Similar to previous observations on the contrasting regulation of GPx1 and TrxR, we found that the TrxR activity is maintained, while GPx1 activity is sacrificed during conditions of inadequate hepatic Se. Several mechanisms that may be responsible for PCB-77-induced hepatic Se depletion are: 1) induction of low molecular weight Se binding proteins that decrease selenoprotein synthesis and facilitate transport of Se or increased extra-hepatic transport of Se via SePP, now recognized as a Se transport protein, 2) alteration of the methylation process resulting in increased selenium excretion via urine, or 3) effect on hepatic selenium metabolism resulting in selenium compounds (selenosugar, inorganic selenium) that may not be biologically useful or available in the liver. Further studies to explore the effects on different selenoproteins and selenium containing compounds by coplanar PCBs and TCDD are necessary to understand the interaction between these environmental contaminants and Se, an essential nutrient as well as a promising cancer chemopreventive agent.

CHAPTER 5: EFFECTS OF SELENIUM ON THE HEPATIC PROMOTING ACTIVITIES OF POLYCHLORINATED BIPHENYLS (PCBS)

Abstract

Polychlorinated biphenyls (PCBs) are persistent organic pollutants that are tumor promoters in the liver. PCBs induce oxidative stress, which may influence carcinogenesis. Epidemiological studies strongly suggest an inverse relationship between selenium and cancers. Despite evidence linking selenium deficiency to hepatocarcinoma and liver necrosis, the underlying mechanisms for selenium cancer protection in the liver remain to be determined.

We examined the effect of dietary selenium on the tumor promoting activities of two PCBs congeners, 3,3',4,4'-tetrachlorobiphenyl (PCB 77) and 2,2',4,4',5,5'-hexaclorobiphenyl (PCB 153) using a 2-stage carcinogenesis model. An AIN-93 torula-based purified diet containing 0.02 (deficient), 0.2 (adequate), and 2.0 mg (supplemental) selenium/kg diet was fed to Sprague-Dawley female rats starting ten days after administering a single dose of diethylnitrosamine (150 mg/kg). Four bi-weekly i.p. injections of either PCB 77 or PCB 153 (150 μmol/kg) were given to the rats after 3 weeks on the selenium diet. Three days before euthanasia, the animals were implanted with osmotic pumps containing bromodeoxyuridine (BRDU).

Our results showed that selenium supplementation did not diminish the induction of hepatic foci by coplanar PCB 77 or ortho-substituted PCB 153. Instead of protection, the number of foci AHF in the liver and number of foci per cubic centimeter of liver among the PCB-77 treated rats was increased as the selenium dietary level increased. Unlike PCB 77, PCB 153 did not show the same selenium dose-response effect nevertheless selenium supplementation did not confer protection against foci development. On the other hand, supranutritional selenium reduced the mean focal volume indicating a possible protective effect by inhibiting progression of preneoplastic lesions into larger foci. Cell proliferation was not inhibited by selenium in the liver of the PCB treated groups. Selenium did not prevent PCB 77 induced decrease of hepatic selenium and associated reduction in GPx1 activity. In contrast, TrxR activity was not affected by the PCBs treatment and by selenium supplementation. These findings indicate

that selenium chemopreventive action in the promotion of hepatic tumors by PCBs is not mediated by GPx1 and TrxR; and a possible anti-cancer action against PCB promoted tumors maybe through reduction of lesion size.

Introduction

The beneficial effect of selenium for cancer chemoprevention has been recognized for nearly nine decades [518]. The selenium supplementation trial by Clark et al. [20,169] primarily designed to prevent skin cancer recurrence, demonstrated that treatment with selenium decreased the risk of cancer of the prostate, lung, and colon and rectum. Although this study and similar clinical trials as well as epidemiologic studies and animal studies pointed to the potential use of selenium for cancer prevention and therapy, the mechanisms by which selenium could protect from cancer has not been well defined. Proposed mechanisms that may explain the anti-cancer effect of selenium involved the antioxidant effect of selenoproteins and source of selenium metabolites that affect carcinogenesis [471]. These mechanisms include, but are not limited to, the antioxidant protection from glutathione peroxidases (GPx) and thioredoxin reductase (TrxR), cell proliferation inhibition, increased apoptosis, effects on the cell cycle, transcription factors and the tumor suppressor gene p53, impaired GSH metabolism, and formation of selenium metabolites that are anti-tumorigenic [117,118,173,188,519].

PCBs are persistent organic pollutants, which have remained widely distributed contaminants because of their environmental mobility and their ability to biomagnify in the food chain [264,520-522]. PCBs were produced and commercially used as mixtures of congeners; there are 209 PCB congeners that have varying toxicities based on the number and position of chlorine molecules around the biphenyl ring. PCBs that have no chlorine substitution at the ortho position can assume coplanar configuration and due to its strong affinity to the aryl hydrocarbon receptor [438] like TCDD are referred to as dioxin-like PCBs [251]. However, PCB congeners that are ortho-substituted do not bind to the AhR and have increased affinity for the constitutive androstane receptor (CAR) [523,524]. Studies have shown that the toxic effects, carcinogenicity and biochemical mechanism of these two groups differ [255,525-527]. Two PCB congeners, one representing each group, were selected for this study: 3,3',4,4'-tetrachlorobiphenyl (PCB-77), a coplanar PCB and Ah receptor agonist; and 2,2',4,4',5,5'-hexachlorobipenyl (PCB-153), a di-ortho substituted PCB and CAR agonist.

Epidemiologic studies have associated PCBs with cancer risk [271,273,277,484] albeit, inconclusively. However, animal studies strongly suggest that PCBs are

carcinogenic [258,315,528,529]. PCB compounds and individual congeners have been found to produce altered hepatic foci in rats [255,357,372,530-533]. PCBs have been shown to act as tumor promoters in animal studies [258,357,534]; recently it has been shown that some PCB congeners and metabolites are possibly cancer initiators [535,536].

Several multi stage-carcinogenicity studies have focused on the prevention of chemical induced hepatocarcinogenesis by dietary selenium; however, none has addressed the potential of selenium in preventing or minimizing PCBs induced tumorigenicity. With the growing popularity of selenium supplementation, it is necessary to understand how selenium interacts with persistent environmental pollutants such as PCBs. Several PCB mixtures and congeners have been known to reduce the activity of GPx1 activity in the liver. Earlier results from our laboratory showed that PCB-77 alone or combined with PCB-153 produced the same effect on the GPx1 activity of rats fed on regular rat chow [317]. Moreover, a concomitant decrease of the GPx1 mRNA transcript and hepatic selenium resulted from the PCB 77 treatment. When we traced the tissue distribution and excretion of selenium in rats fed with regular diet for rodents that contain adequate selenium, we found that the PCB 77 effect on selenium levels may be due to enhanced excretion of selenium in the urine (unpublished results). Therefore, it was of interest to determine whether selenium supplementation would overcome the effect of PCBs on the hepatic selenium and GPx1 activity leading to better antioxidant capacity in the liver. Ultimately, we expected that supranutritional selenium will prevent or minimize the hepatocarcinogenic effects of PCBs. The aim of this study was to determine the chemopreventive effect of dietary selenium on the tumor promoting potentials of two PCBs congeners: coplanar, Ah-receptor-mediated, efficacious CYP1A inducer PCB 77 and a non-coplanar, CYP2B-inducing PCB 153 using an initiation-promotion hepatocarcinogenesis model.

Materials and Methods

Chemicals

PCB-77 and PCB-153 were synthesized and characterized as described previously [411]. Both compounds had a purity of > 98% as determined by gas chromatography. Stripped corn oil used as vehicle for the PCBs was procured from Acros Organics (Morris Plains, NJ). All dry constituents of the AIN-93 purified diet were from Harlan Test Diets (Madison, WI). The anti-PGST antibody was purchased from Novocastra Laboratories Ltd. (Newcastle, England). The Vectastain staining kit was from Vector Laboratories (Burlingame, CA). The sodium selenite (Na_2SeO_3), Thioredoxin (Trx) from E.coli and all other chemicals were from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Experimental design and animal treatment

This study was approved by the University of Kentucky Institutional Animal Care and Use Committee. Weanling, female Sprague-Dawley rats, weighing 120 grams, were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed three rats per cage in a temperature- and light-controlled room.

The initiation-promotion protocol is shown in Figure 5.1. After one week of acclimatization, all rats were injected p.o. with diethylnitrosamine (DEN) dissolved in saline (150 mg DEN/kg). After a 10-day recovery period, rats were randomly divided into three diet groups (27-28 per group) and fed a purified diet (Table 5.1) based on the AIN-93 diet formulation [537] ad libitum until the rats were euthanized. Selenium (as Na_2SeO_3) was mixed with the diet at a dose of 0.02 , 0.2, and 2.0 mg selenium/kg diet corresponding to low, adequate and high selenium diet, respectively.

After three wks, randomly grouped rats (9-10 rats/treatment/diet) were injected i.p. with corn oil, PCB-77, or PCB-153 (300 μmol/kg). The rats received 3 additional biweekly i.p. injections and were euthanized 10 days after the last injection. Three days prior to euthanasia, Alzet osmotic pumps containing BrdU (20 mg/ml, 10 μL/hr) were implanted on the rats as described [391]. Rats were sacrificed using carbon dioxide asphyxiation followed by cervical dislocation. Liver pieces were removed and fixed in

10% buffered formalin. The remaining liver was frozen in liquid nitrogen and stored at −80 °C.

Table 5.1: Composition of AIN 93-based Purified Diet

Constituent	*Percent of Diet*
Torula yeast	30
Corn starch	36
Dextrose monohydrate	19.95
Cellulose fiber	5
AIN-93M mineral mix, without selenium	3.5
AIN-93M vitamin mix	1
Choline bitartrate	0.25
DL-methionine	0.3
Soybean oil	4

Tissue processing and BrdU-placental glutathione S-transferase (PGST) immunostaining

At the time of necropsy, liver slices from all lobes were cut and fixed in formalin followed by paraffin embedding. The sections (6 μm) were stained using a BrdU/PGST double immunohistochemical staining method [538] with modification using a Vector laboratories protocol. Briefly, the sections were dried, deparaffinized and rehydrated in a series of one Hemo De and three alcohol changes. After blocking the endogenous peroxidase with 3% hydrogen peroxide in methyl ethanol, the sections were immersed in Citra (antigen retrieval) and boiled three times for 5 minutes each in a microwave oven at 10% power. The sections were incubated in normal horse serum before applying avidin and biotin. Sequential treatment with anti-BrdU primary antibody, biotinylated anti-mouse IgG, and ABC solution were followed with diaminobenzidine (DAB) staining for BrdU labeled nuclei. The slides were treated with goat serum prior to applying avidin/biotin. This was followed with sequential incubation with PGST primary antibody, biotinylated anti-rabbit IgG, and ABC-AP. Vector Red stain mix containing alkaline phosphatase substrate was applied followed by counterstaining with hematoxylin. The section was dehydrated in a series of absolute alcohol prior to mounting.

Quantitation of PGSTi-positive altered hepatic foci

The number and volume of PGSTi-positive altered hepatic foci were measured using a quantitative stereology computer program, STEREO, as described previously [223,391,539-542]. Briefly, utilizing a microscope (Nikon Eclipse E800), images of the stained liver section were taken and processed with a Scion Image software, Microsoft Photoshop and background correction program to generate data on tissue outline, X-Y coordinates, focal transaction diameter and location. Foci were defined as a cluster of at least 6 hepatocytes that were positively stained for PGST. The data was exported and organized using the Build New Data File program where the focal information was edited and verified against the microscope image. From the quantitative stereology program, we used the resulting files for each rat and diet-treatment group for data calculation and combination. Only foci with 128 micron diameter were used to calculate volume % of foci in liver (Delesse), number of foci/cm^3 (Saltykov), number of foci/liver (Saltykov), and mean volume of foci (Saltykov) for each rat and group.

Counting of BrdU-stained nuclei

Representative images of all liver sections (2 images/lobe) were taken and processed with the Scion image program and Photoshop (Figure 2). The NLIA program, a component of the STEREO program [542,543] was employed to automatically count the BrdU labeled nuclei in each image. The magnification of the images used was 20x and a total of approximately 4,000-6000 nuclei were counted per slide. Cells that had brown nuclei were identified as BrdU labeled. All labeled and total hepatocytes in the non-focal area were counted. The labeling index was the percentage of number of labeled nuclei per total nuclei counted.

Protein assay

Protein concentration of supernatants, dialysates and cytosolic fractions was determined using the BCA method (Pierce Chemical Company). Briefly, 200 µl of mixed reagent, 1 part Reagent A (Cu^{+1}) and 50 parts Reagent B (bicinchoninic acid), was added to each well of a 96-well plate. 10 µl of each diluted sample or bovine gamma globulin standard (0. 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml) was added to each well in duplicate. After

incubating the plate for 30 minutes at 37°C, the plate was cooled to room temperature and read at 562 nm with a Bio-Tek plate reader. Using the KinetiCalc software, the concentration of protein (mg/ml) was calculated for each sample based on the standard curve.

Homogenate and dialysate preparation

Frozen liver tissues (0.45-5.0 g) were homogenized in phosphobuffered saline (PBS) pH 7.4 with 1 mm EDTA solution for 30 seconds using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 1300 g for 30 minutes and the supernatant collected for dialysis. The protein concentration of each supernatant was adjusted to 8 mg/ml protein with the PBS-EDTA solution prior to dialysis. The supernatant was dialyzed in PBS pH 7.4 to remove endogenous GSH. Approximately 1 ml of supernatant was pipetted into prepared dialysis tubing, clipped to close, and placed into a beaker containing PBS solution (100 ml PBS/1 ml supernatant) for 16 hours at 4°C. The buffer was replaced at least one time. The dialysate was collected and aliquoted. The protein concentration of the dialysate was adjusted to 1 mg protein/ml [11,544].

Thioredoxin reductase (TR) activity assay

The TrxR activity was determined using a method of Holmgren and Bjornsted [494] as modified by Hill et al. [11]. Briefly, a reaction mixture is prepared containing 1 ml of 10 mg/ml insulin, 400 μl of 1M HEPES buffer (pH 7.6), 80 μl of 0.2M EDTA and 80 μl of 40mg/ml freshly prepared NADPH. 60 μl of the reaction mixture was added into tubes. Two tubes were allocated to each dialysate sample. To one tube, 15 μl of 60 μmol E. coli thioredoxin (test) was added while 15 μl of distilled water was added to the other tube (control) to represent the non-TR-thioredoxin system-dependent reaction. After adding 105 μl (≈105 μg protein) of dialysates into the test and control tubes, the reaction was incubated at 37°C for 15 minutes. The reaction was stopped by adding 750 μl of 0.4 mg/ml DTNB (5,5'-dithiobis(2-nitrobenzoic acid) in 6 mol guanidinium hydrochloride and the absorbance of both test and control mixtures were measured at 412 nM. The TR-thioredoxin system-dependent NADPH reduction of insulin was determined by

subtracting the absorbance of the control from that of the test reaction mixture. TrxR activity was expressed as A_{412} units x 1000/(min x mg protein).

Cytosolic fraction preparation

Approximately 0.5 gram liver was used in the preparation of liver homogenate as previously described [447]. Briefly, the liver was homogenized in 0.25M sucrose/0.1mmol ethylenediaminetetraacetic acid (EDTA), pH 7.4 using an Ultra-Turrax homogenizer, then centrifuged at 10,000xg for 20 minutes. The supernatant was collected and then centrifuged at 100,000xg for one hour. After separating the cytosolic fraction (supernatant) from the microsomal pellet, the cytosolic fraction was aliquoted for protein determination and enzyme assay.

Glutathione peroxidase activity assay

The glutathione peroxidase activity of the cytosolic fraction was determined using the method of Paglia and Valentine [199] as modified by St. Clair and Chow [449]. Briefly, a reaction mixture of 2 mmol EDTA, 2.5 mol sodium azide (NaN_3), 0.2 mmol NADPH, 1 mmol GSH, 1 unit/ml glutathione reductase in 50mmol Tris-HCl (pH 7.6) was prepared. In a quartz cuvette, 10 μl of cytosolic fraction diluted with Tris-HCl ($\approx$10-20 μg protein) and 940 μl reaction mixture was pre-incubated for 1 min at room temperature. 30% H_2O_2 was added into the reaction cuvette and mixed. The decrease in absorption was monitored spectrophotometrically for 60 seconds (10 readings at 6 seconds interval) at 340 nm. For total glutathione peroxidase activity, 25 μl cumene hydroperoxide (1.2 mmol) is rapidly added and absorbance is monitored for 1 minute at 340 nm. The enzyme activity was expressed as nmoles NADPH/min/mg protein..

Selenium determination

Liver samples were analyzed using the neutron activation analysis (NAA). Briefly, the samples were weighed and freeze-dried. An aliquot of the freeze-dried sample was irradiated for 7 seconds at a flux of approximately 5 x 10^{13} ncm^{-2}sec^{-1} , decayed for 15 seconds and counted for 25 seconds. The samples were analyzed using the gamma-ray (Energy = 161.9Kev) from the decay of Se-77m (half-life = 17.45 sec and

52.4% abundant). The standard comparator method was used to obtain the absolute Se concentration. In addition to the HPGe detector, the spectrometer system included a Tennelec 244 coupled to a Canberra 599 loss-free counting module and a Canberra 9660 DSP. Data acquisition and peak extraction were done using VAX Station 3100 model 38 Canberra ND application software. Selenium was expressed as mg selenium/kg wet tissue.

Statistical analysis

Results were analyzed by two-way ANOVA followed by Tukey post-hoc test for comparison of group means. The comparisons of the PCB treatment groups with the corn oil (control) groups with respect to the number of foci/cm^3 and number of foci/liver were statistically tested using a Negative Binomial Regression model with logarithm as the link function as previously described [404]. Briefly, Pearson χ^2 value adjusted for over dispersion was used to assess the goodness of fit while the method of maximum likelihood was used to estimate the parameters. Wald's asymptotic procedure determined the *p* values for the significance of the differences between the PCB treatment groups and the control groups. The PROC GENMOD of SAS, version 8 software was used to perform the statistical analyses.
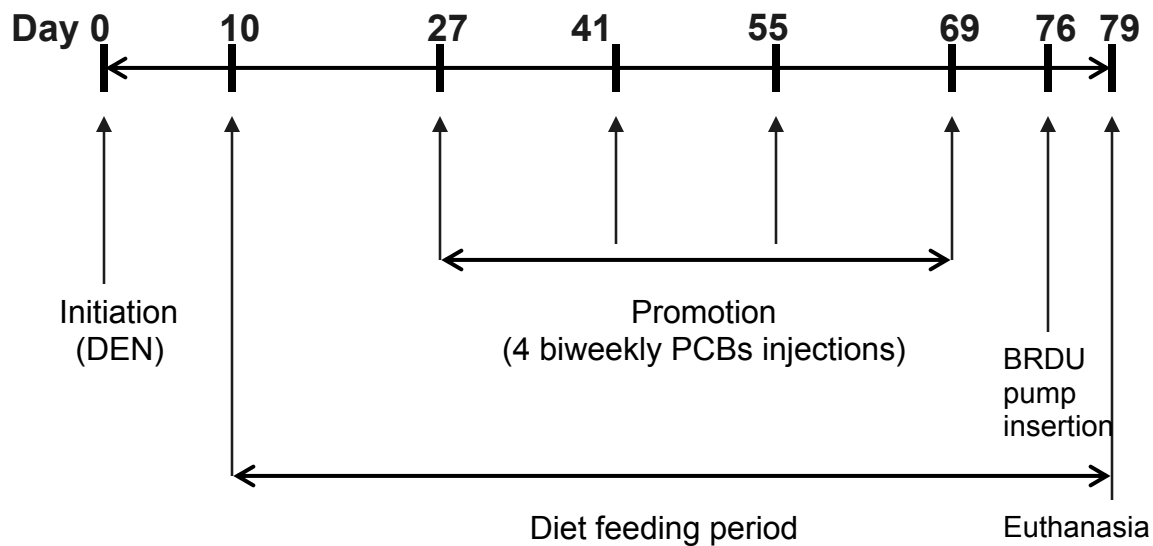
Figure 5.1: Initiation-promotion protocol. Female Sprague-Dawley rats were initiated with DEN (150 mg/kg body weight p.o) before feeding with AIN 93-based purified diet containing varying levels of selenium (0.02, 0.2, 2.0 mg Se (as Na_2SeO_3)/ kg). The promotion period consists of four biweekly i.p. injections of corn oil, PCB-77 or PCB-153.

Results

Effect on Body Weights and Relative Liver Weights

The liver weights and the relative liver weights (as percentage of body weight) were significantly increased in rats treated with PCB-77 or PCB-153 at all levels of selenium, with the highest increase seen in the PCB-77 groups (Table 5.2). In contrast, PCBs had no effect on final body weight or weight gain (not shown). Selenium did not affect the body weight or the gross or relative liver weights.

Effect on Altered Hepatic Foci Number and Volume

The number and volume of altered hepatic foci (AHF) were quantified using placental glutathione S-transferase (PGST) as an immunohistochemical marker. The total number of PGST-positive foci and the number foci per cm^3 were significantly increased in rats treated with PCB 77 compared with the corresponding corn oil group in each diet. Surprisingly, this PCB 77 effect significantly increased with increasing dietary selenium intake and this was especially notable in the high selenium diet groups (Figure 5.3a and b). The percent of the liver that was occupied by PGST positive foci was also significantly increased in the PCB-77-treated rats; however, the high Se diet group was not increased when compared with the adequate Se group (Figure 5.3c).

In PCB-153 treated rats, the number of PGST positive foci per cm^3 and per liver were not affected by selenium. Similarly, although the focal volume fraction was increased by PCB-153 treatment, it was significant only for the group fed with adequate selenium but not with high selenium. Selenium supplementation produced a 3-fold increase in the number of AHF per liver and per cm^3 in the corn oil group compared to the adequate selenium group (Figure 5.3a and b). In contrast, the mean focal volume of the PCB-77 treated groups was not increased compared to the corresponding control whereas PCB-153 treatment produced a significant increase only in the low and adequate Se diet groups. The adequate selenium diet did not have an effect in decreasing the effect of the PCBs seen in the selenium deficient group. However, for the high selenium diet groups, although the PCBs treated groups did not differ from the vehicle group, the mean

focal volume was drastically reduced by 3 fold compared to the levels seen in the adequate group (Figure 5.3d).

Effect on Cell Proliferation

Cell proliferation in non-focal areas was measured using BrdU labeling index. BrdU was incorporated into DNA during DNA synthesis through a 3-day infusion of BrdU using an osmotic pump. The labeled nuclei are the cells that progressed through the S phase of cell cycle. PCB treatment slightly increased the BrdU labeling index of the hepatocytes surrounding the AHF in all selenium diet groups; however, this effect was not statistically significant except in the PCB-153 treated group that received adequate selenium (Figure 5.4).

Effect on GPx1 Activities

A significant dose dependent increase in the activity of the cytosolic selenium-dependent glutathione peroxidase (GPx1) was observed in relation to selenium diets (Figure 5.5). At supranutritional dose of 2 ppm selenium, GPx1 activity continued to increase. In the PCB-77 treated groups, a 3 fold decrease in GPx1 activity was noted in the selenium deficient group. Adequate selenium diet produced more than 2-fold significant increase in the GPx1 activity of PCB 77 compared to the low selenium diet group with the same treatment, PCB-77 effect in reducing GPx1 activity compared to the corresponding control remained significant. In contrast selenium supplementation did not lead to a further increase in the GPx1 activity. In general, PCB-153 also reduced the GPx1 activity in all the diet groups, but this effect was significant only in the high selenium group. For both PCBs, the trend are similar in that adequate selenium in the diet contributed to increasing the GPx1 activity compared to the low selenium group. Further selenium supplementation did not produce any further increase in the activity.

Effect on TrxR Activities

The TrxR activity in the dialyzed supernatant of the PCBs treated groups was slightly higher in the low (both PCBs) and adequate (PCB-77) selenium groups, but this effect was not significant (Figure 5.6). In contrast, a non significant decrease in TrxR

activity was seen in the high dose selenium groups treated with PCBs. There was no significant difference seen between the selenium diet groups. Compared with the adequate selenium group, rats dosed with PCB 77 and corn oil fed with selenium deficient diet had 25% less TrxR activity.

Effect on Hepatic Selenium

A significant dose dependent increase in the hepatic selenium can be seen between the low dose selenium (0.02 ppm), the adequate dose (0.2 ppm) and the high dose (2 ppm) groups regardless of treatment (Figure 5.7). This trend was also observed in the GPx1 activity of the control groups but not for the PCBs groups. Interestingly, although the selenium levels increased correspondingly as selenium dietary intake was increased, this did not diminish the effect of PCB 77 in significantly depressing selenium levels compared to the corresponding control groups. On the other hand, although the same trend was noted for the PCB 153 treated groups, only the high selenium diet group showed a significant reduction in hepatic selenium compared to the control.

Table 5.2: Effect of selenium and PCBs on body and liver weights

Selenium	Treatment	Liver weight	Body weight	LW/BW ratio
Low	corn oil	9.74 ± 0.70	258.11 ± 9.75	3.75 ± 0.16
	PCB-77	14.36 ± 0.50[a]	246.67 ± 4.56	5.82 ± 0.17[a]
	PCB-153	11.22 ± 0.18[a]	262.80 ± 4.46	4.40 ± 0.11[a]
Adequate	corn oil	9.30 ± 0.19	254.78 ± 3.88	3.64 ± 0.05
	PCB-77	13.19 ± 0.38[a]	240.44 ± 5.43	5.50 ± 0.15[a]
	PCB-153	11.58 ± 0.57[a]	252.90 ± 5.16	4.41 ± 0.15[a]
High	corn oil	9.86 ± 0.27	251.00 ± 3.82	3.93 ± 0.08
	PCB-77	13.70 ± 0.46[a]	246.22 ± 2.97	5.56 ± 0.14[a]
	PCB-153	12.29 ± 0.40[a]	258.80 ± 4.23	4.74 ± 0.11[a]

Results are expressed as mean $\pm$ SEM. Each group contained 9-10 animals. [a]values are significantly different from their respective controls treated with corn oil ($p < 0.05$).
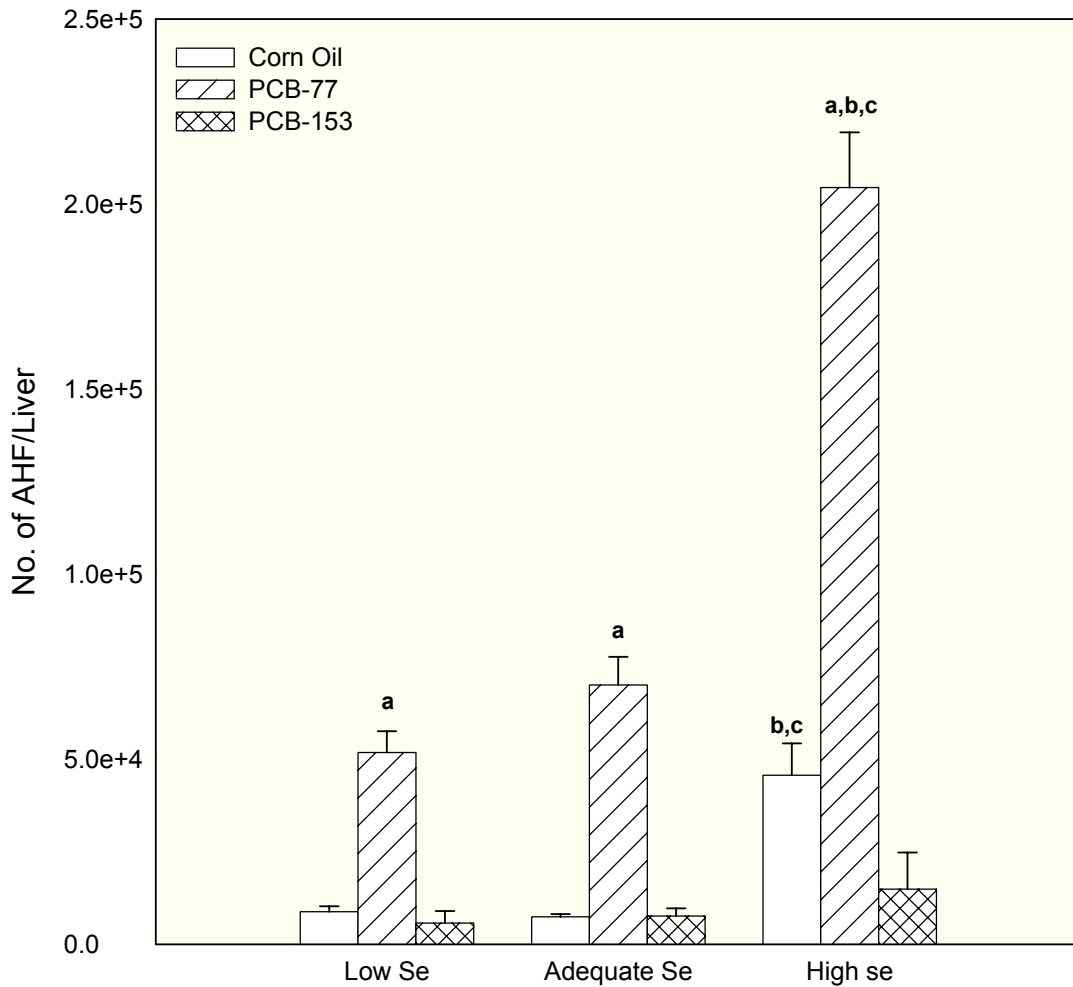
Figure 5.3a: Number of PGST-positive foci per liver. Results are expressed as mean ± SEM. Each group contained 8-10 animals. [a]Values are significantly different from their respective controls treated with corn oil ($p < 0.05$). [b]Values are significantly different from the low selenium diet group with similar treatment ($p < 0.05$). [c]Values are significantly different from the adequate selenium diet group with similar treatment ($p < 0.05$)
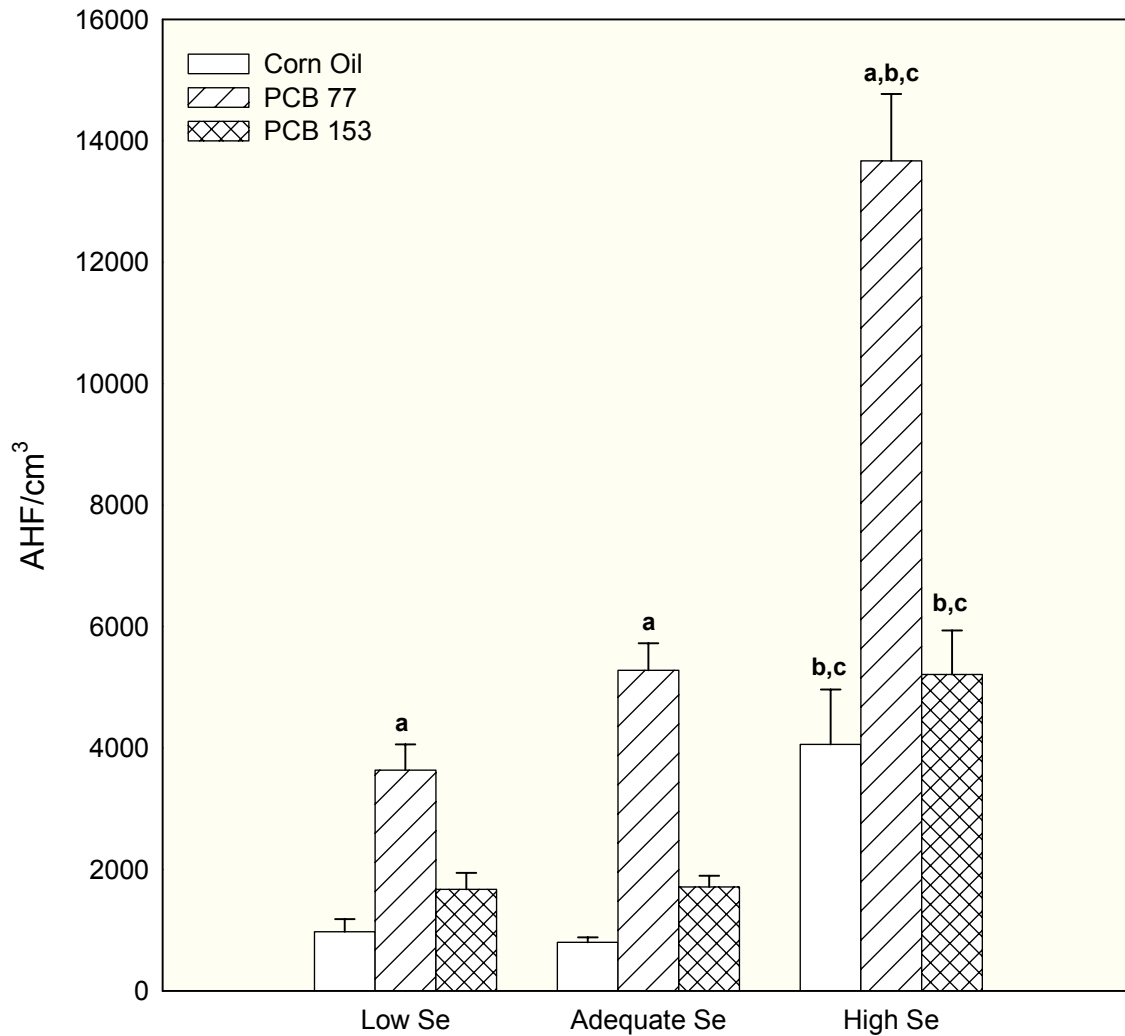
Figure 5.3b: Number of PGST-positive foci per cubic centimeter. Results are expressed as mean ± SEM. Each group contained 8-10 animals. [a]Values are significantly different from their respective controls treated with corn oil ($p < 0.05$). [b]Values are significantly different from the low selenium diet group with similar treatment ($p < 0.05$). [c]Values are significantly different from the adequate selenium diet group with similar treatment ($p < 0.05$).
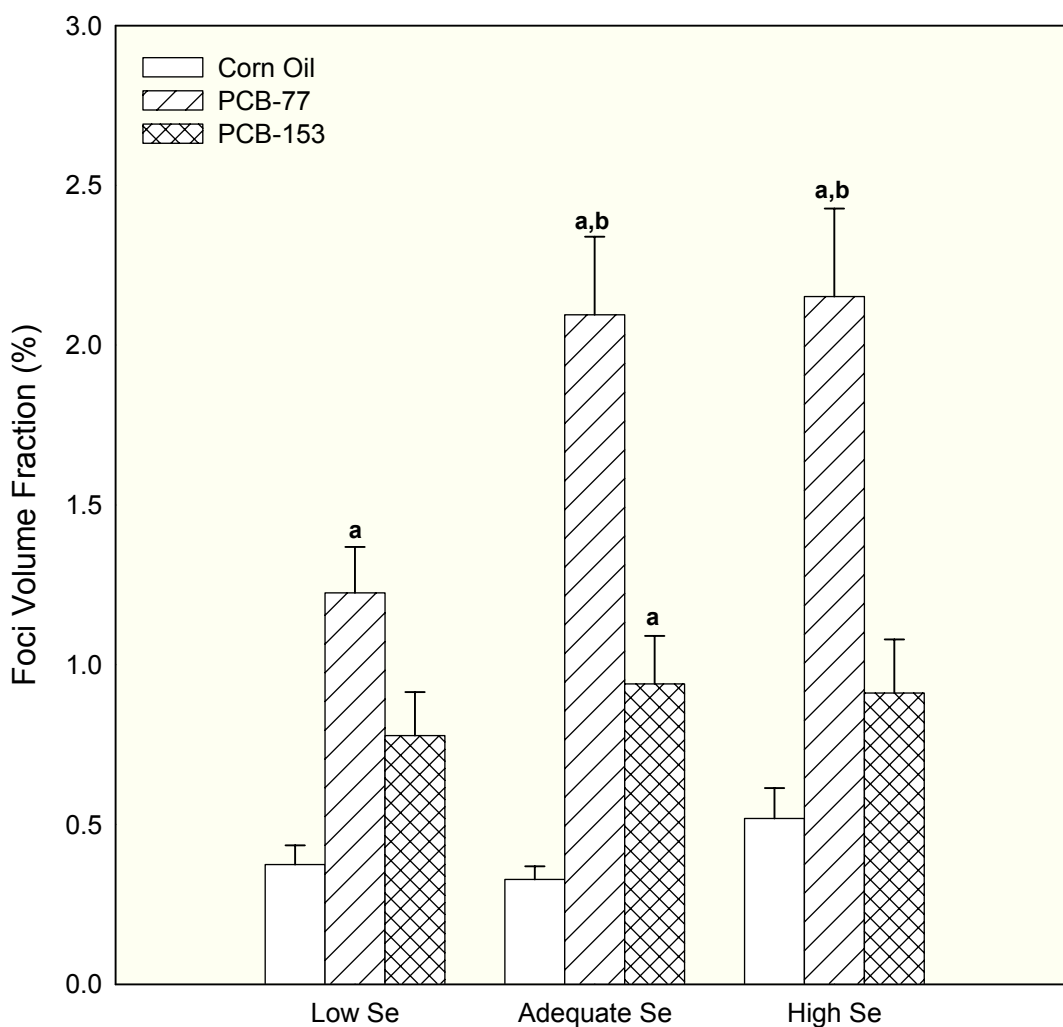
Figure 5.3c: Percentage of the liver occupied by PGST-positive foci. Results are expressed as mean ± SEM. Each group contained 8-10 animals. [a]Values are significantly different from their respective controls treated with corn oil ($p < 0.05$). [b]Values are significantly different from the low selenium diet group with similar treatment ($p < 0.05$). [c]Values are significantly different from the adequate selenium diet group with similar treatment ($p < 0.05$).
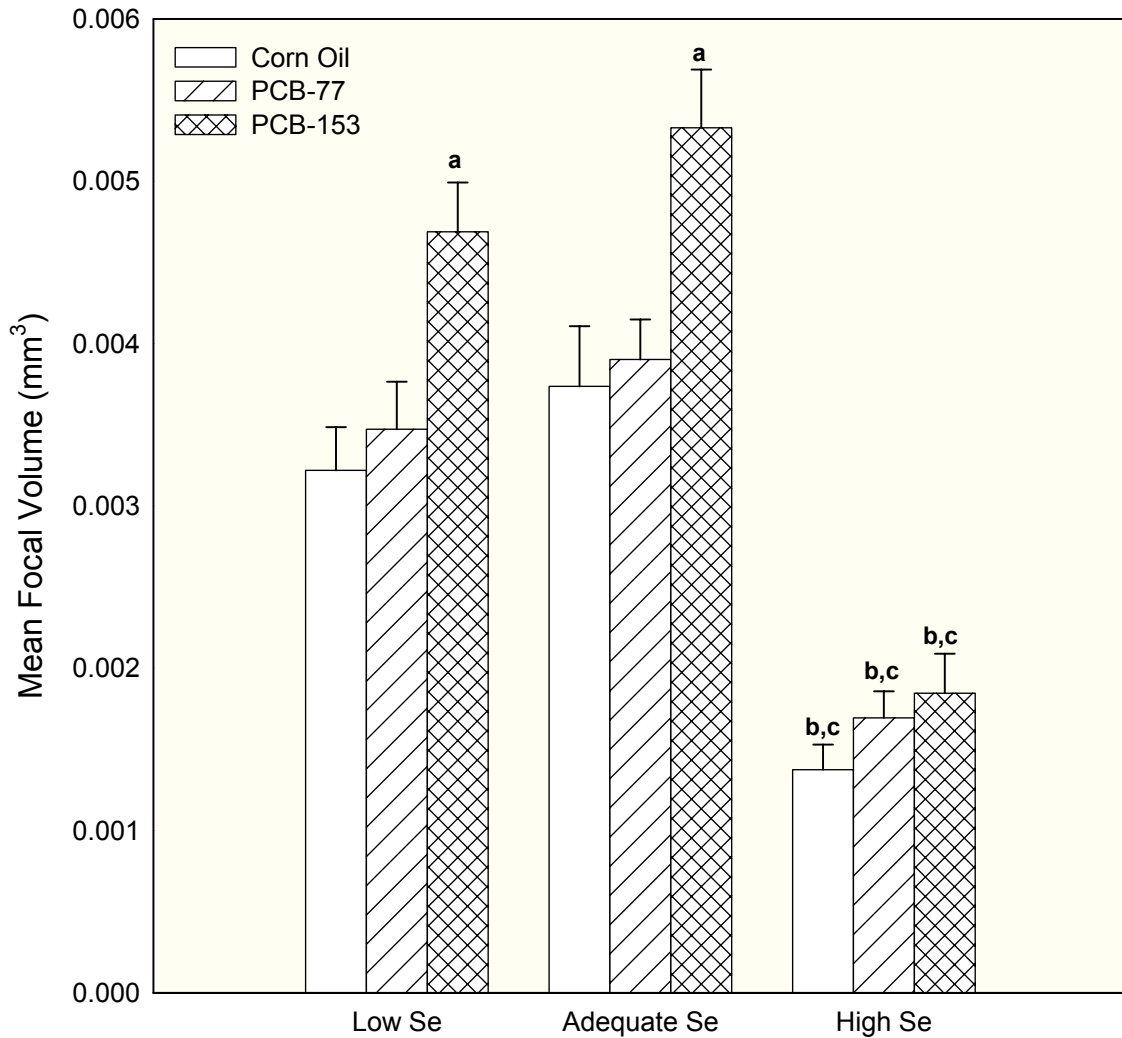
Figure 5.3d: Mean focal volume of PGST-positive foci. Results are expressed as mean ± SEM. Each group contained 8-10 animals. [a]Values are significantly different from their respective controls treated with corn oil ($p < 0.05$). [b]Values are significantly different from the low selenium diet group with similar treatment ($p < 0.05$). [c]Values are significantly different from the adequate selenium diet group with similar treatment ($p < 0.05$).
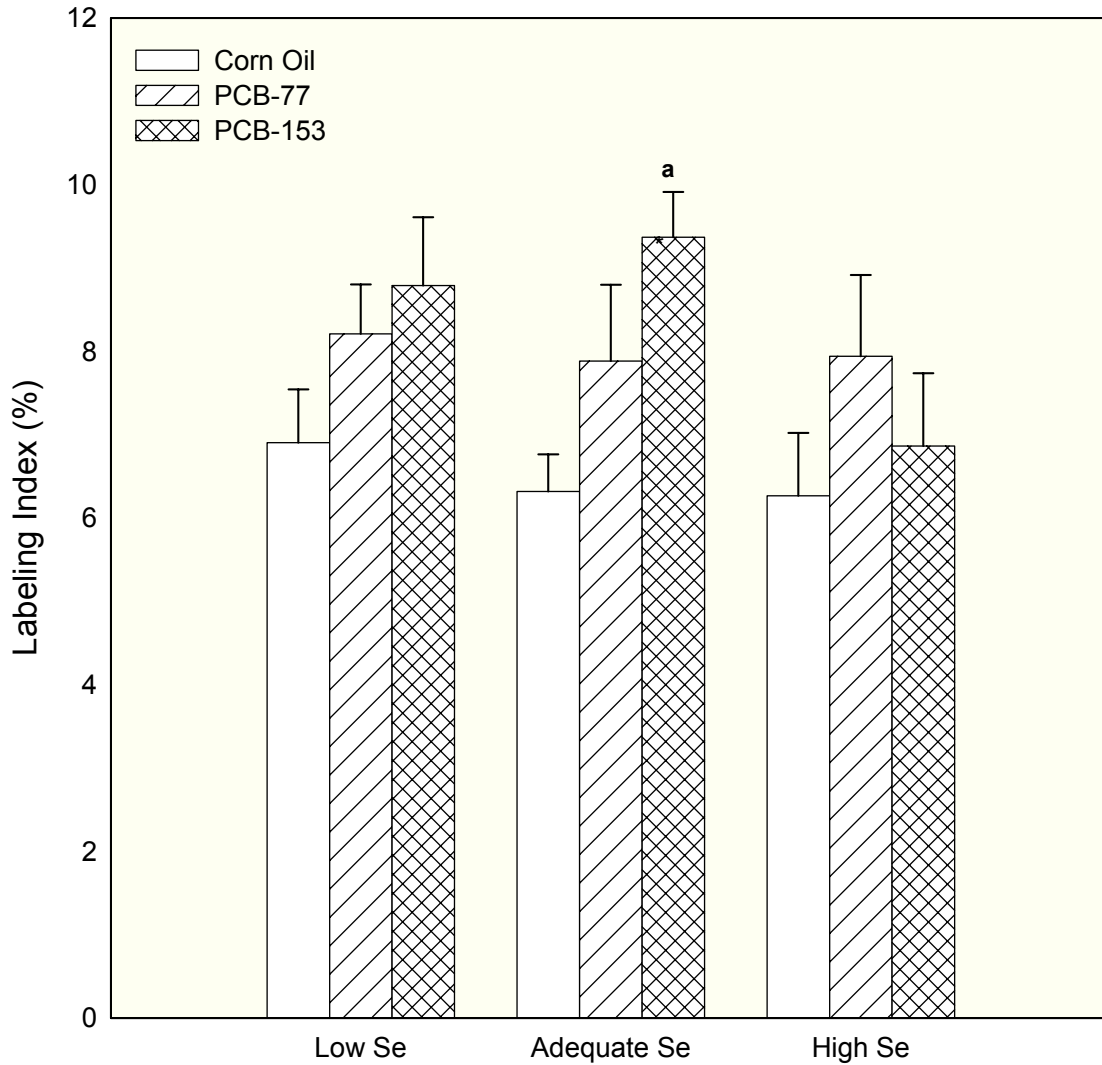
Figure 5.4: Cell proliferation in rat hepatocytes. Results are expressed as mean ± SEM. Each group contained 8-10 animals. [a]Values are significantly different from their respective controls treated with corn oil ($p < 0.05$).
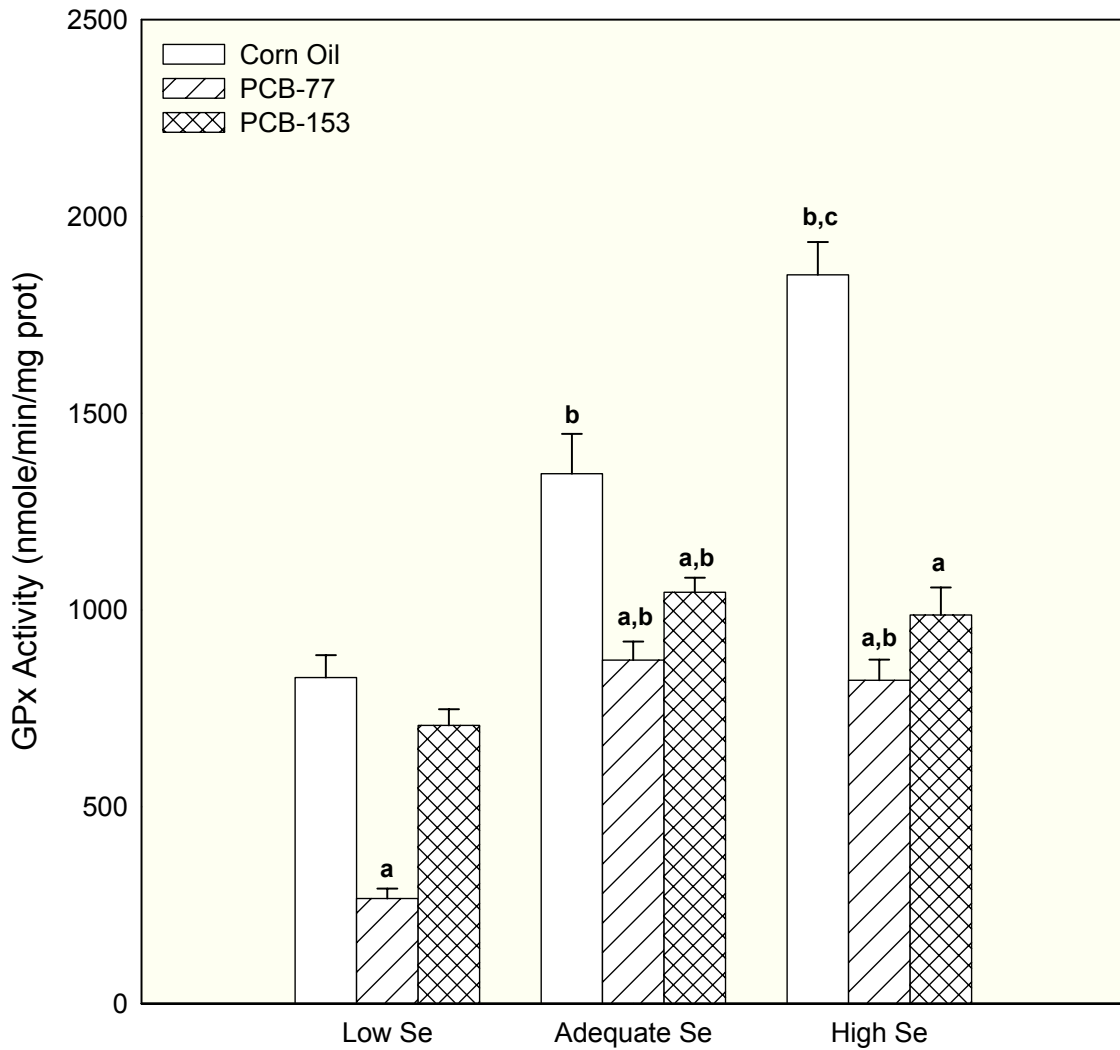
Figure 5.5: Glutathione peroxidase activity. Results are expressed as mean ± SEM. Each group contained 8-10 animals. [a]Values are significantly different from their respective controls treated with corn oil ($p < 0.05$). [b]Values are significantly different from the low selenium diet group with similar treatment ($p < 0.05$). [c]Values are significantly different from the adequate selenium diet group with similar treatment ($p < 0.05$).
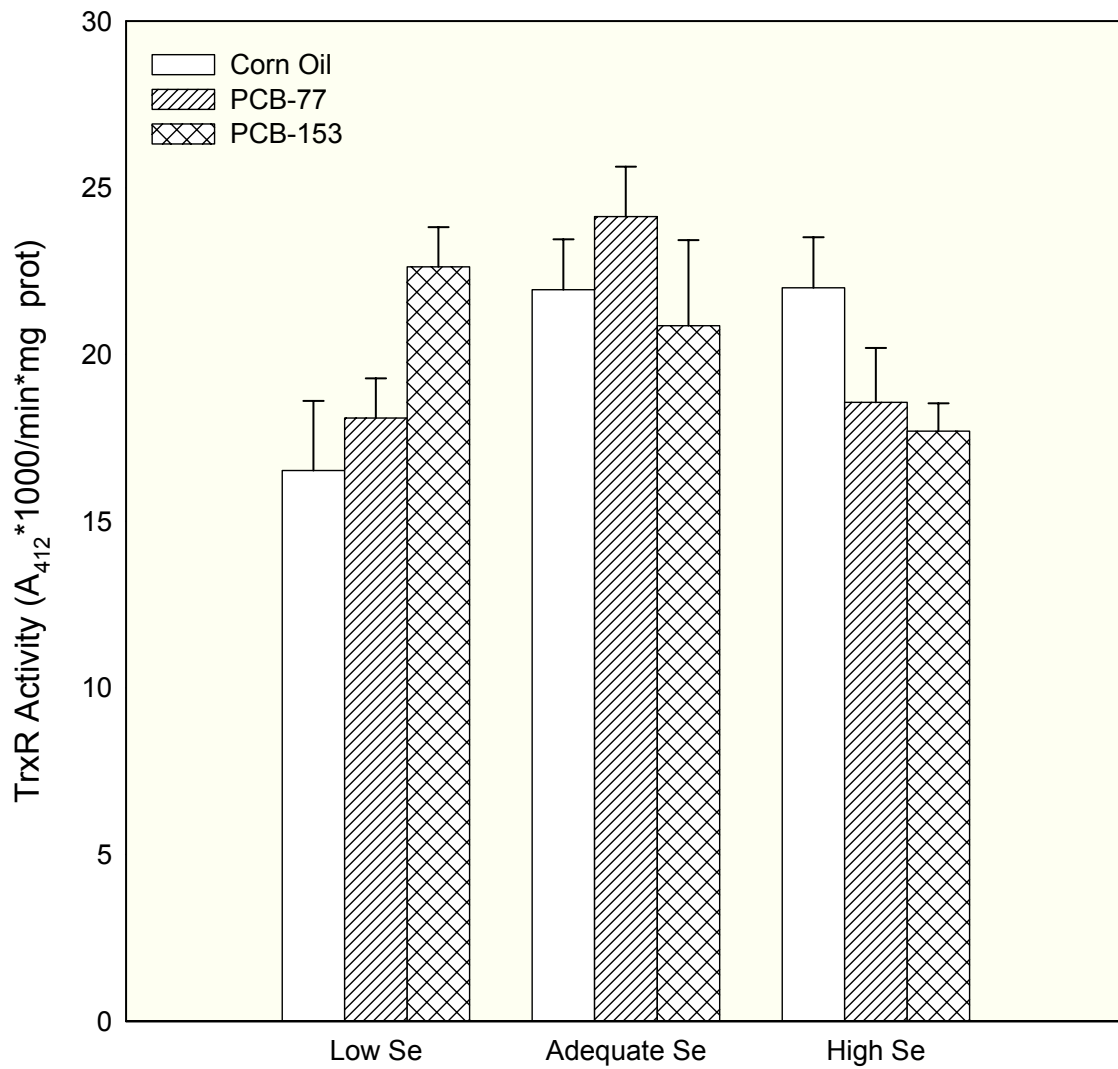
Figure 5.6: Thioredoxin reductase activity. Results are expressed as mean ± SEM. Each group contained 8-10 animals.
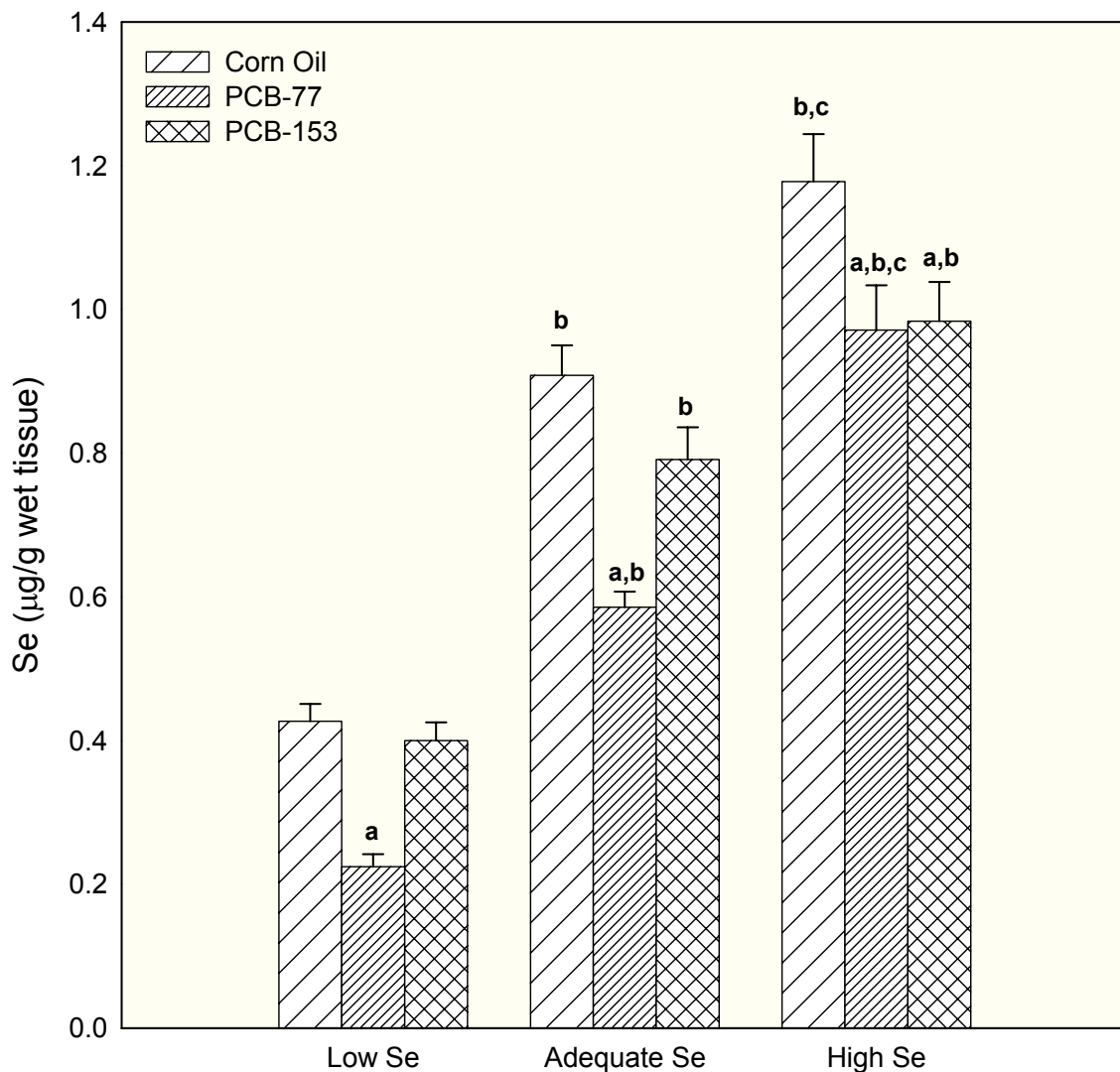
Figure 5.7: Hepatic selenium concentration. Results are expressed as mean ± SEM. Each group contained 8-10 animals. [a]Values are significantly different from their respective controls treated with corn oil ($p < 0.05$). [b]Values are significantly different from the low selenium diet group with similar treatment ($p < 0.05$). [c]Values are significantly different from the adequate selenium diet group with similar treatment ($p < 0.05$). Selenium levels were measured using the NAA method.

Discussion

PCBs are known carcinogenic chemicals that have been shown to decrease both Se levels and GPx1 activity in the liver of experimental animals [317,410,411,413,462]. The ability of PCB 77 and PCB 153 to promote hepatocarcinogenesis in DEN initiated animals are demonstrated by several studies [258,384,391,488,545]. The chemopreventive effects of Se against cancer is proven in animal studies and has been supported by epidemiological and supplementation trials [1,117,120,173,185,546]. In the present study, we show that supranutritional Se diet did not inhibit PCB 77 and PCB 153 induction of preneoplastic lesions in the rat liver. Instead, the PCB 77 induced number of PGST positive foci per cm^3 and per liver significantly increased with increasing dietary Se level notably in the high Se diet group, however the focal volume fraction of the high Se diet group was not further increased compared with the adequate Se group. Similarly Se did not affect PCB-153 effect on the number of PGST positive foci per cm^3 and per liver although the focal volume fraction was increased by adequate Se intake but not by high Se dietary intake. In contrast, high Se diet drastically decreased the mean focal volume of both PCBs as well as the control group. Se dietary intake had no effect on cell proliferation in liver of both PCBs except in the adequate Se, PCB 153 treated rats. GPx1 and Se levels were reduced in the liver by PCB 77 but not PCB 153, however thioredoxin reductase (TrxR) activity was not affected by either PCBs. The differential effect on TrxR and GPx1 by PCBs study confirmed the contrasting regulation of GPx1 and TrxR as a result of dietary Se.

AHF or putative preneoplastic lesions are used as marker in hepatocarcinogenesis studies of carcinogens. Most hepatocarcinogenesis models use the resistant hepatocyte models [227], which employs partial hepatectomy (PH) followed by an initiating agent, diethylnitrosamine (DEN) and then promotion with a known promoting agent such as 2-acetylaminofluorine (2-AAF). The 2-stage or initiation-promotion model, such as the protocol employed in this study, is also used to study potential or known tumor promoters such as PCBs. In this study, the promotion phase consisted of multiple dosing with PCBs as the promoting agent.

Comparison of our findings with prior hepatocarcinogenesis studies on selenium is limited by differences in experimental systems including hepatocarcinogenesis model,

gender and species of rodents, form and dose of dietary selenium, and tumor markers [233-237,242,547]. For example, studies have shown that the initiating agent DEN or PH plus DEN themselves contribute to focal development. Moreover, the degree of selenium protection against hepatic tumorigenesis is affected when Se is administered before or during the initiation phase [230,242]. In contrast, Baldwin and Parker [236] reported that Se affected focal formation when given during promotion and not initiation. In this study, to ensure that the Se supplementation effect was directed towards the PCBs induced tumor promotion phase we fed the animals ten days after DEN initiation and throughout the promotion phase.

Several studies have investigated the effect of selenium on different phases of hepatocarcinogenesis using varying in-vivo hepatocarcinogenesis protocols, initiating agents and tumor promoters. Two studies demonstrated the lack of Se effect on induction of hyperplastic hepatic nodules or carcinoma incidence whether Se supplementation was given during initiation or promotion stages or all throughout study. In these studies, they employed a 2-stage hepatocarcinogenesis model using DEN as initiator and PB as tumor promoter [231,232]. Similarly, our findings showed that 0.2 and 2.0 ppm Se did not confer protection against PCB 77 or PCB 153 tumor promoting effects in relation to the number of PGST positive foci per liver as well as per cm^3, and fraction of the liver occupied by foci (Figure 5.3a, b and c), but 2.0 ppm Se was effective in reducing the mean volume of foci of both PCBs and control rats. Conflicting result on focal development was also reported by Baldwin and Parker [236]. 2-AAF treated rats supplemented with 6.0 ppm Se resulted in decreased mean volume and focal volume fraction of gamma-glutamyl-transpeptidase (GGT)- positive foci without affecting the number of foci/cm3 of liver Similarly using a Solt-Farber protocol, 1 and 5 ppm Se administered to rats had no effect on the number of hepatic nodules during initiation and the fraction occupied by nodules in the liver of tumors during the 3-month 2AAF-progression stage; however, Se decreased the volume density or the fraction occupied by the nodules in the liver during the promotion and 6-month progression stages [233].

Other hepatocarcinogenesis studies have shown that selenium inhibits focal growth [234,235,238]. Lei et al. [237] demonstrated that Se is effective against AFB1-induced cancer by reducing H&E altered foci at the initiation and promotion phases and

inhibiting progression of the nodules into hepatocellular carcinoma; however, more number of foci and larger nodules developed in the 6 ppm Se compared to the 3 ppm Se male rats. In comparison, our findings indicate that 2 ppm Se supplementation enhanced PCBs-induced generation of PGST positive foci (increased number per liver and per cm^3 and volume fraction) that are smaller in size (reduced mean volume) compared to the 0.2 ppm Se whereas the mean volume of the 0.2 ppm Se did not differ from the control.

The nature of the anticarcinogenic effects of Se remains unclear. Likewise, the mechanism by which Se protects from hepatic cancer is not known. Several mechanisms have been proposed including cell proliferation inhibition and induction of apoptosis [548,549]; altered carcinogen metabolism; and antioxidant protection. PCB 77 had been shown to depress hepatic selenium and GPx activity [317]. Since selenium metabolism occurs mainly in the liver, it follows that decreased available selenium will lead to decreased available Se for selenocysteine incorporation or selenoprotein synthesis [49]. The synthesis of different selenoproteins follow a certain hierarchy and GPx1 production has been shown to be less important compared to TrxR or iodothyronine deiodinases [11,173,193,241,550,551]. The reactive oxygen species (ROS) produced by PCBs metabolites, which lead to oxidative damage is one mechanism believed to cause tumor promotion [357]. Therefore, the PCB-induced selenium depression leading to reduced anti-oxidant defense may be amplifying the growth of new preneoplastic lesions resulting from oxidative damage. In addition, another PCBs effect is glutathione depletion. Selenite reacts with GSH to form selenodiglutathione, GSSeSG, which could produce oxidative stress via redox cycling of the GSSe$^-$ anion [552-554]. GSSeSG is also the precursor of the primary intermediate, hydrogen selenide, which is then methylated into methylselenol, the key selenium metabolite that has been demonstrated to possess anti-cancer properties [120,188,552,555]. Studies have argued that selenite causes oxidation and depletion of intracellular GSH, which is more cytotoxic than pro-apoptotic [218,554,556]. The combined effect on GSH by both PCBs and Se may have contributed to decreased methylselenol production leading to diminution of the focal size.

LeBeouf et al. [234] also noted Se (6 ppm) effects on focal growth (mean volume) with no corresponding effect on the number of "preneoplastic" lesions in the liver indicating that supplemental Se may be inhibitory to the progression of PCB-induced

preneoplastic lesions to carcinoma. Since triphenylselenonium chloride produced smaller lesions and fewer large-sized lesions but did not affect pre-malignant mammary intraductal proliferations, it was suggested that selenium action may be more cytostatic than inhibitory of proliferation [184]. Furthermore, it was shown that Se inhibited the progression of AFB1-induced nodules to hepatocellular carcinoma [237].

Coplanar PCB 77 has been shown to strongly induce the generation of hepatic foci compared with the ortho-substituted PCB-153 [391]. Hence, unlike PCB 77, PCB 153 did not show the same Se dose-response effect on focal development. Our findings may have been influenced by the use of young adult female rats. It was reported that young adult, female Sprague-Dawley rats are more sensitive to DEN-induced cancer effects [232]. Previous studies also showed that female rats are more sensitive to PCBs effects [315,317].

Cell proliferation in the liver is important in the carcinogenic process of initiation, promotion and progression [557]. One of the mechanisms for Se chemoprevention that has been proposed was inhibition of cell proliferation [234,558-560]. Using the same initiation-promotion protocol but without Se intervention, female Sprague-Dawley rats given 300 μmol/kg weight PCB-77 showed increased cell proliferation in both focal and non-focal hepatocytes [391]. Hence, we expected the chemopreventive property of Se to decrease the ability of PCBs to promote tumor through increased cell proliferation. In this study, we found that DNA synthesis in the non-focal hepatocytes of PCBs-dosed rats was not significantly affected by adequate or supranutritional Se. However, we found, albeit insignificantly, that the labeling index was consistently higher in the PCBs treated groups compared with their corresponding controls except for the PCB-153 rats fed with adequate Se. In contrast, a study found that cell proliferation in the surrounding, non-nodular tissue was significantly increased in the Se supplemented rats although the opposite was observed in the nodules [233]. The conflicting results support the possibility that inhibition of cell proliferation may not be a common mechanism by which Se affords cancer protection against carcinogenesis. Combined with the increased number and fraction of foci, this also raises the question of whether hepatocyte proliferation is a condition for PCB-induced growth of DEN-initiated cells.

Protective mechanisms produced by Se metabolites, namely selenodiglutathione, selenide and methyl selenol, are proposed to be responsible in part for the anti-cancer effects of Se, but we can not discount the antioxidant function of selenoproteins [7,196,208,440,561,562]. Two major selenoenzymes, GPx and TrxR, are essential components of the two major redox systems in the cell, the glutathione and thioredoxin systems. It is known that cytosolic GPx1 has low affinity for Se incorporation when Se is limiting. In addition, GPx1 protein synthesis and expression are drastically decreased in Se deficiency whereas other selenoproteins are not as affected [514]. The finding that GPx1 knockout mice did not develop abnormalities when subjected to hyperoxic condition led to the belief that another mechanism compensates for the loss of GPx1 function [202]. The premise that GPx prevents carcinogenesis remains an issue because GPx activity was found to be at its maximum in animals with adequate dietary Se whereas anti-cancer effects are mostly observable at supranutritional levels [117,191,193,562-566]. Se deficient animals exposed to carcinogenic chemicals have been shown to be more sensitive to development of 8-OHdG DNA adducts leading to oxidative damage [174]. One mechanism for PCBs toxicity is the generation of ROS leading to oxidative stress [348,424,491]. The PCB congeners used in this study both induce cytochrome P-450 enzymes as well as increased lipid peroxidation [335]. As discussed earlier, PCB mixtures and some congeners depress Se dependent glutathione peroxidase (GPx1) activity. We observed that indeed glutathione peroxidase activity was strongly reduced by PCB-77 especially in rats fed with Se-deficient diet. Although PCB 153 did not display as strong an effect as PCB-77, it also depressed GPx1 activity in rats fed adequate and supplemented Se diets. Overall, Se supplementation was not able to prevent reduction of the glutathione peroxidase activity by PCBs.

It was observed that Se inhibited the incidence of ciprofibrate-induced altered hepatic foci in rats and this effect correlated with increased GPx1 activity in the serum and the liver [238]. However, Se did not decrease the oxidative damage indices, such as TBARs and conjugated dienes, indicating that increased GPx activity does not necessarily confer protection against oxidative damage. The lack of correspondence between GPx1 activity and oxidative stress is shown when hepatoma cells injected into Sprague Dawley rat livers decreased the GPx1 activity but did not significantly affect

oxidative stress, as measured by TBARs and 8-hydroxy-2'-deoxyguanosine [239]. Another study also demonstrated that high levels of selenite intake do not inhibit oxidative damage; instead high selenium intake (2.0 ppm as sodium selenite) increased the concentration of 8-OHdG in liver DNA produced by high levels of DEN, indicating that the anti-cancer effects of selenium may not be through prevention of oxidative damage [241]. In contrast, it was shown that Se supplementation of DEN-initiated rats decreased lipid peroxidation in liver tissues [240]. Furthermore, upon Se supplementation, lipid peroxidation levels in tumor tissue of cancer-bearing organs were decreased. Our study found that the number of altered hepatic foci was increased in the control group receiving supranutritional Se even though GPx1 activity was increased. This point to the possibility that supranutritional Se may be enhancing PCB 77 -induced tumor development.

Thioredoxin reductase (TrxR) catalyzes the NADPH-dependent reduction of thioredoxin (Trx). The activated Trx controls cellular redox processes such as transcription (activation of NF-κB), protein-DNA interactions, embryonic development, and DNA synthesis. Some studies demonstrate the protective role of the Trx system in cancer; however, indications that this system may also have pro-tumorigenic effects has been noted [117]. Trx has been shown to inhibit apoptosis which therefore favors tumor growth [117,210,567,568]. It was observed that rats fed with high Se (1.0 ppm as sodium selenite) diet had a 2-fold increase in hepatic TrxR activity although there was no accompanying increase in TrxR protein [569]. However, after long term feeding with high Se diet, hepatic TrxR activity eventually decreased to the level of the control, which they attributed to a decrease in TrxR protein synthesis resulting from decrease in Se incorporation. This may explain why the high Se diet in this study failed to produce a corresponding increase in the TrxR activity. Furthermore, Gallegos et al. [517] noted that selenite treatment did not affect TrxR1 mRNA stability or protein possibly because an increase in TrxR mRNA level occurred first, followed by increase in protein levels and then increased activity. Studies have shown that GPx1 and TrxR are regulated differently [189,204,515]. Using TGFα/*c*-myc mice, a model of accelerated hepatocarcinogenesis, it was shown that GPx1 expression was decreased in tumors compared to the surrounding normal tissue [204]. In contrast, TrxR expression and activity were increased in tumors.

The opposing regulation of TrxR and GPx1 was confirmed in human prostate cell lines from normal and cancer cells where it was shown that GPx1 was repressed while TrxR was increased in tumor cells compared to the normal cells. In our study, we did not differentiate the enzyme activity in the foci versus the surrounding normal cells; however, the apparent repression of GPx1 activity by PCB 77 is strongly associated with the increased number of foci per liver and increased focal volume ratio. In contrast, TrxR activity was not affected by the PCBs treatment. Moreover, although high dietary Se increased hepatic Se associated with a corresponding increase in GPx1 activity, TrxR activity was not affected.

Our findings on hepatic Se indicate that the effect of PCBs on the Se levels is associated with GPx1 activity. A dose-related increase in hepatic Se level was observed for both PCBs and control. Again, PCB 77 has stronger reducing effect on hepatic Se compared with PCB 153. This result confirms a previous finding that PCB 77 suppression of GPx activity is associated with its reducing effect on hepatic Se[317]. Our group traced the distribution and excretion of Se after a single i.p. dose of PCB-77 and found that PCB-77-induced depletion of hepatic Se may be due to enhanced Se excretion in urine (unpublished result).

Otter et al. [570] observed that DNA synthesis between severely Se deficient rats (0.01 ppm Se as sodium selenite) and Se adequate rats (0.33 ppm) were not different; however, after pretreatment with hepatopoietin or partial hepatectomy, a 3-fold increase in cell proliferation was observed in the Se-deficient rats suggesting that induction of DNA synthesis may have occurred due to severe hepatic stress. Another study used partial hepatectomy in its carcinogenesis model and yet the cell proliferation in the DEN+AAF group that received Se deficient diet did not differ from the adequate Se (1 ppm Se) group, indicating that cell proliferation may not be a function of hepatic stress resulting from partial hepatectomy [233]. However, within tumors or nodules, this study observed that cell proliferation was decreased by Se. One could argue that this result may be due to the observed increased TrxR activity in the tumors as previously reported [204]. Our findings indicate that the TrxR activity was not affected by Se adequate or supplemented diets pointing to the need for further studies to elucidate the role of TrxR in hepatocarcinogenesis..

In summary, our findings showed that Se supplementation magnified the PCB-77-induced generation of altered hepatic foci in terms of number and volume fraction. On the other hand, Se supplementation reduced the mean focal volume of the foci. Cell proliferation inhibition may not be one of the mechanisms by which Se confers protection against PCBs induced tumor promotion. Se supplementation did not prevent PCB 77 induced decrease in hepatic Se which was accompanied by a corresponding reduction in GPx1 activity. In contrast, TrxR activity was not affected by the PCBs treatment or Se supplementation.

CHAPTER 6. DETERMINATION OF SELENIUM IN RAT LIVER, FECES AND URINE BY ZEEMAN-GRAPHITE FURNACE ATOMIC ABSORPTION SPECTROMETRY: COMPARISON WITH NEUTRON ACTIVATION ANALYSIS AND FLUOROMETRIC METHODS

Abstract

Selenium (Se) is an essential element that has chemopreventive properties against cancer. Evaluation and monitoring of Se status and toxicity associated with dietary intake of Se require the determination of biological markers or indices such as Se concentration in serum, plasma, red blood cells, hair, nails, urine or feces. Several analytical methods are available for measuring trace concentrations of Se (ng/g) in biological samples. For total Se, the methods being used include inductively coupled plasma mass spectrometry (ICP-MS), gas chromatography, neutron activation analysis (NAA), fluorometry and graphite furnace atomic absorption spectrometry (GFAAS). This study is aimed at determining Se concentration of rat liver, urine and fecal samples employing Zeeman-GFAAS together with a closed microwave digestion technique, and at comparing the results of the GFAAS method with values produced by the NAA and fluorometric methods. Results indicate that the analysis of liver tissues, urine and feces with Zeeman-GFAAS after closed microwave digestion produced lower Se values compared with the NAA and the classical fluorometric method and possible causes may be incomplete digestion, loss of Se after digestion or loss of Se during the pre-atomization stage.

Introduction

Selenium (Se) is an essential trace element that has cancer chemopreventive effects [20,170,173,185,188,472]. In addition, supranutritional levels of Se are believed to be associated with enhanced immune response, less serious viral infections, and reduced cardiovascular risk [571]. The effect of severe Se deficiency in humans is best exemplified by Keshan disease, a cardiomyopathy among young children and women of child bearing age and by Kashin-Beck disease, characterized by bone deformity [4-6]. On the other hand, excess Se can be toxic. Se intoxication in humans tends to be accidental, as a consequence of accidents or errors in the formulation of a Se-supplemented diet [16,52]. In animals, Se toxicity has been observed as a result of consumption of seleniferous plants.

The growing use of Se as nutraceutical has raised concerns about the appropriate dietary intake. In addition, new synthetic seleno-compounds are being introduced as substitutes to natural Se forms [120,572]. Therefore, the need to evaluate and monitor Se status and toxicity associated with dietary intake of Se and seleno-compounds requires the determination of biological markers or indices, such as Se concentration in serum, plasma, RBC, hair, nails, urine or feces. Moreover, it is prudent to determine the bioavailability of Se in food sources to know whether high absorption and retention will lead to accumulated concentrations in the body that could pose a hazard or would be beneficial to special groups needing Se supplementation, such as populations in Se-deficient areas.

Several analytical methods are available for measuring trace concentrations of Se (ng/g) in biological samples. The choice for the appropriate method will depend on whether total Se or individual Se metabolites will be determined. For total Se, the methods being used include inductively coupled plasma mass spectrometry (ICP-MS) gas chromatography, neutron activation analysis (NAA), fluorometry, and graphite furnace atomic absorption spectrometry (GFAAS). The AAS method has been modified to improve detection limits, specificity, precision and accuracy. The modifications included the use of Zeeman background correction and hydride generation. Fluorometry remains a reliable method for determining Se; however, the limitations are in the sample

preparation. The NAA method has lower Se detection limits; however, this can only be done in institutions which have a nuclear reaction capability [573].

From small experimental animals, biological samples such as tissues, whole blood or plasma, urine, and feces presents a problem not only because the available quantity is limited, but also because intense matrix effects can cause background interference which could compromise accuracy and precision of the results. Hence, consideration for the quantity and type of sample matrix is necessary when deciding the type of sample preparation required for the analytical method to be used. The closed vessel microwave digestion technique requires a small amount of sample and offers a cleaner and safer alternative to other preparation methods, such as the hot plate digestion, dry ashing, high-pressure ashing, and open vessels microwave digestion.

This work describes a procedure for analyzing rat liver, urine and fecal samples using GFAAS with Zeeman background correction together with microwave digestion. The results of the GFAAS method are compared with those from the neuron activation analysis and a fluorometric method.

Experimental Methods

Graphite furnace atomic absorption spectrometry

Instrumentation

The GFAAS used consists of a Model SpectrAAZeeman-800 graphite furnace atomic absorption spectrometer (Varian, USA) equipped with a GTA-100 power supply and a programmable, automatic sample dispenser. The AAS has background correction based on the Zeeman effect with a transversal electromagnetic field.

Reagents

All chemicals were purchased from Sigma-Aldrich Chemical Co. (USA) unless otherwise indicated. All reagents used were of analytical-reagent grade, and water was double distilled (specific resistivity 18). Se for AAS stock solution (1 mg/ml) in 2% v/v nitric acid of 99.999% purity was diluted as necessary to prepare the standards. The automated sampler was programmed to use a blank and three Se standards using a 100 µg/l bulk Se standard solution. Se reference stock solution (1 mg/ml)) was obtained from SPEX (USA) and 5 and 10 µg/l reference Se solutions (SPEX) were prepared and used as internal standards. The standard reference material (NIST-SRM 1577 freeze-dried bovine liver) for Se was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA). Palladium (Pd) modifier, (10 g/L) in 15% nitric acid for graphite furnace-AAS was obtained to prepare a chemical modifier for Se.

Sample Collection

Urine and feces were collected from female Sprague Dawley rats fed with an unrefined diet (Purina rodent laboratory chow, Purina Mills, St. Louis, MO). The animals were kept in metabolic cages, one animal per cage. Feces and urine of each individual rat were collected daily in polyethylene tubes and weighed (feces) or measured (urine volume). Urine was centrifuged for 10 minutes to remove any rat chow contaminants before 2 ml aliquots were placed into polypropylene tubes. At the end of one month, the animals were euthanized and livers were excised and weighed. All samples were stored in a -80 C freezer in aluminum foil (liver and feces) or polypropylene tubes (urine).

Approximately 2 grams of feces from each animal (four animals per group) was pooled, freeze dried, ground, mixed well and split into 0.5 g samples. For urine samples, approximately 4 ml were taken from each animal, thawed, pooled, mixed well, and split into 1 ml aliquots and freeze-dried. Due to the high salt content of urine, freeze drying took at least 24 hours. Duplicate split samples were used for each Se determination method.

Closed Microwave Digestion of Samples

Approximately 0.2 to 0.5 gram wet liver tissue was weighed and placed into an insulated, Teflon microwave digestion vessel (UDV-10). The freeze-dried urine samples were first reconstituted in 0.5 ml double deionized water and then pipetted into digestion vessels. Approximately 0.7-1.0 gram ground, freeze-dried feces were weighed into digestion vessels. Two ml pure grade concentrated nitric acid was added to each digestion vessel containing the liver and urine samples; for fecal samples 4 ml nitric acid was used. The digestion vessels were tightly sealed with a Teflon cover equipped with a pressure release vent. The samples were digested using a computer controlled microwave digester) employing different programs for each sample matrix (Table 6.1). At the end of the digestion, the acid digest was allowed to cool, transferred into polyethylene centrifuge tubes, and was immediately diluted with deionized water to make up a 6-10 ml volume.

GFAAS Se Operating Protocol

The GFAAS default program set by the manufacturer was used with some modifications [574]. The operating conditions are shown in Table 6.2. The study used a Se high intensity, boosted discharge, hollow cathode lamp (Varian UltrAA) and pyrolytically coated partitioned graphite tubes. Argon was set at a flow-rate of 3.0 min^{-1} during all stages, except the atomization stage. The selenium calibration curve was prepared by programming dilution of a 100 ng Se/ml standard to concentrations ranging from 5-40 ng Se/ml. Blanks consisted of 20% nitric acid. A 500 mg/l Pd solution in 1% citric acid and 1% nitric acid was co-injected with the sample to prevent Se from evaporating during the ashing stage [450]. A 1% nitric acid rinse solution with 0.1 % Triton X-100 and 0.01 % Antifoam B was used to rinse the micro-sampler. Peak height

was recorded and used to determine the absorbance response. Standard calibration, blank and 5 or 10 ng Se/ml internal control solution was run at the beginning and after every 8 samples. The final Se concentration of the sample was calculated using Se concentration (μg/L) of the 5 μl, the sample volume and the wet liver weight.

NAA analysis of Se

The NAA analysis was undertaken at the University of Missouri Research Reactor Center (Columbia, MO). Briefly, the liver, feces and urine samples were analyzed using the gamma-ray (Energy = 161.9Kev) from the decay of the short-lived Se-77m (half-life = 17.45 sec and 52.4% abundant). The samples were irradiated for 7 seconds using a thermal neutron flux of approximately 5×10^{13} nθcm^{-2}θsec^{-1} , decayed for 15 seconds and counted for 25 seconds. Standard comparator method was used to obtain the absolute Se concentration. Replicate standards and quality control materials (NBS SRM 1577 bovine liver certified at 1.1 ppm for Se) were analyzed at the beginning and end of the analysis. The activated samples were analyzed with a spectrometer system that included a HPGe detector, a Tennelec 244 coupled to a CANBERRA 599 loss-free counting module and 9660 DSP. Data acquisition and peak extraction were done using aVAX Station 3100 model 38 application software.

Fluorometric determination of Se

Se was determined using the fluorometric method of Olson et al. [575,576] modified by Cantor et al. [577,578]. Briefly, approximately 0.5 gram samples were weighed and digested overnight in trace metal grade concentrated perchloric and nitric acid. Using a microkjeldahl digester, the partially digested samples were wet ashed in culture tubes using nitric and perchloric acids. The ashed samples were then titrated to a pH of 1.5 with NaOH and HCl and buffered with ammonium hydroxide EDTA before adding 5.0 ml diaminonaphthalene (DAN). Selenium reacts with DAN to form a fluorescent piazselenol, which is then extracted from the solution with cyclohexane. The fluorescence of the resulting extract was read at 360 nm excitation and 520 nm emissions. A standard curve (linear regression equation) was prepared using triplicate readings of standard Se solutions containing 0, 0.1, 0.2, 0.3 and 0.4 μg Se. Se concentrations were

then calculated using the regression equation and the sample weight. The assay results were verified against NIST-SRM 1577b bovine liver reference standard (0.073 ± 0.006 µg Se/g).

Table 6.1: Digestion program using CEM MSP 1000 Microwave Digester

Sample Matrix	Stages:	1	2	3	4
Wet Tissues	Pressure (psi)	050	100	150	
	Time (min)	10	10	10	
Urine	Pressure (psi)	040	080	120	
	Time (min)	8	8	14	
Feces	Pressure (psi)	040	080	120	160
	Time (min)	8	8	20	20

Table 6.2: GFAAS conditions for Se analysis

Program Parameters	
Element - Matrix:	Se
Instrument Type:	Zeeman
Instrument Mode:	Absorbance
Calibration Mode:	Concentration
Measurement Mode:	Peak Height
Replicate standard and samples:	3
Wavelength:	196.0 nm
Slit Width:	1.0 nm
EHT:	289 Volts
Lamp Current:	15.0 am
Background Correction:	BC On
STANDARD 1:	5. ug/L
STANDARD 2:	10 ug/L
STANDARD 3:	30 ug/L
Reslope Lower and Upper Limit:	75.0 %, 125.0 %
Recalibration Rate:	10
Calibration Algorithm:	Linear Origin
Cal. Lower and Upper Limit:	20.0 %, 150.0 %
Total Volume:	25 uL
Sample Volume:	20 uL
Modifier:	500 ppb Palladium in 1 % Citric acid
Modifier Vol.:	5 uL
Rinse solution:	1% HNO_3 in 0.1 % Triton X-100 and 0.01 % Antifoam B

Stages	Step	Temp (C)	Time[493]	Flow (L/min)
Drying	1	85	5.0	3.0
Drying	2	95	50.0	3.0
Drying	3	120	12.0	3.0
Ashing	4	1300	11.0	3.0
Ashing	5	1300	10.0	3.0
Ashing	6	1300	2.0	3.0
Atomization	7	2600	0.8	0.0
Atomization	8	2600	2.0	0.0
Cleaning	9	2800	2.0	3.0

Results and Discussion

Several spectrometric techniques are used to analyze Se levels in biological samples that have been prepared by various methods [450,579,580]. However, most methods require large amounts of samples for digestion. In this study, we digested approximately 0.5 gram rat wet liver tissue, 2 ml freeze-dried urine and 0.4 gram freeze-dried fecal samples using a closed microwave digestion method. Se was then analyzed using a Zeeman-GFAAS. The same samples were also analyzed by NAA and fluorometric methods. The results are shown in Table 6.3. The Se concentration values of the GFAAS analyzed wet liver tissues, freeze-dried feces and urine, and NIST-SRM bovine liver standards were all lower than the values produced by the NAA and fluorometric methods. The concentrations were particularly very low for the liver samples. It is possible that loss of Se occurred during the digestion process as a result of incomplete digestion or Se evaporation; or during the GFAAS ashing stage, where the Pd modifier may not be sufficiently binding selenium to prevent Se volatilization. Sabe et al. [581] found that using atomic fluorescence detection, the recovery of the major selenium metabolite in urine, trimethylselenonium ions (TMSe), is very low (5%) due to poor conversion of this species into inorganic selenium during the microwave digestion when a mixture of nitric acid-hydrogen peroxide was used; however, the use of nitric acid-sulfuric acid produced a higher recovery. Our study used nitric acid alone to digest the samples. Using microwave energy, closed vessel digestion with mineral acids in PTFE vessels, which this study used, was reported to produce incomplete digestion compared with quartz vessels. The advantage of closed vessels is that it is safer, cleaner, contamination is minimized, and sample decomposition is rapid. Loss of Se may also occur when the vessel is opened while not sufficiently cooled.

Table 6.3: Comparison of the results obtained for Se using Zeeman-GFAAS, NAA and Fluorometric methods

	Zeeman-GFAAS Method		Neutron Activation Analysis		Fluorometric Method	
	AVG	*STD DEV*	*AVG*	*STD DEV*	*AVG*	*STD DEV*
Feces 1	0.225	0.004	0.291	—	0.406	0.012
Feces 2	0.263	0.007	0.547	—	0.405	0.009
Feces 3	0.235	0.005	0.431	—	0.477	0.004
Feces 4	0.222	0.036	0.669	—	0.453	0.004
Liver 1	0.084	0.006	1.15	0.070	1.221	0.030
Liver 2	0.045	0.003	1.78	0.284	1.044	0.014
Liver 3	0.075	0.010	1.22	0.054	1.280	0.075
Liver 4	0.136	0.027	0.98	0.029	1.030	0.016
Urine 1	0.047	0.004	0.17	0.004	1.495	0.057
Urine 2	0.058	0.007	0.13	0.003	1.400	0.038
Urine 3	0.050	0.001	0.16	0.014	1.670	0.029
Urine 4	0.080	0.001	0.16	0.001	1.655	0.028

Triton X-100 is a non-ionic surfactant that is used as diluents to whole blood samples to produce a more homogeneous solution and decrease interfacial tension between sample and graphite [574]. Triton X can produce variable atomization results and build-up of a carbonaceous crust inside the graphite tube; however, Triton X in this study was used only in the rinse solution and was not mixed with the samples. Since the samples were in 10%-20% nitric acid solution, we used a 10% nitric acid solution for blanks and standard dilutions. However, diluents were not added to the digested samples before GFAAS analysis.

Modifiers are added to bind selenium and prevent loss during the pre-atomization stage [574]. The samples were co-injected with Pd modifier, as recommended by the manufacturer although nickel could also be used a modifier although some studies pre-injected the Pd modifier into the graphite tube before the sample is introduced. The effect

of pre-injection or co-injection of Pd modifier to results has not been determined in this study.

We also noted excessive background absorption and residue build-up inside the graphite tube for all the samples and excessively so with the urine and fecal acid digests. To avoid contamination between samples, we increased the temperature setting of the graphite furnace cleaning stage to 2800°C. The reproducibility of the samples when analyzed with the GFAAS method in triplicate on three non-consecutive days was not very satisfactory (data not shown); hence, we need to further conduct recovery studies using several modifications of the digestion process and to establish quality parameters, i.e. precision data in terms of repeatability.

In conclusion, the Zeeman-GFAAS method after closed microwave digestion produced lower Se values compared with the NAA and the classical fluorometric method. Possible causes may be incomplete digestion, loss of Se after digestion or loss of Se during the pre-atomization stage. Since the Zeeman-GFAAS coupled with closed microwave digestion would be a safer and cheaper method for analyzing selenium and an accessible alternative compared to the NAA and fluorometric methods especially in laboratories that have GFAAS capability, ways to improve selenium recovery by modifying some digestion and/or operating parameters will be studied in the future.

CHAPTER 7. SUMMARY, CONCLUSIONS AND FUTURE STUDIES

Summary and Conclusions

Polychlorinated biphenyls (PCBs) are persistent organic pollutants that are complete carcinogens, tumor initiators and tumor promoters in rodent liver [251,255,258,315,357,582]. PCBs, like PCB 77 and PCB 153, induce drug metabolism and promote oxidative events within the cell, which may influence carcinogenesis [258,357,412,424,583,584]. Selenium (Se) is an essential micronutrient that has been well studied for its anti-cancer activities; however, the mechanisms for its chemopreventive action remain to be elucidated [52,94,117,118,173,471,472,585]. Epidemiological studies strongly suggest an inverse relationship between Se status and cancers[1,20,170,185,439,586]. Exposure to environmental contaminants has been shown to lead to a Se deficient status [52,445]. PCBs produce reactive oxygen species like hydrogen peroxide, which in turn are detoxified by glutathione peroxidases (GPx) [22,369,587,588]. PCBs reduce the activity of GPx and recently it has been shown that gene expression, mRNA for GPx, and Se content of the liver were also diminished [317]. We hypothesized that certain PCB congeners affect selenium metabolism in the rat liver resulting in diminished antioxidant capacity of selenoproteins (GPx and thioredoxin reductase), which could alter the ability of Se to protect against PCBs induced oxidative stress and tumor promotion.

In the first study, we determined the influence of a coplanar PCB, PCB 77 (3,3',4,4'-tetrachlorobiphenyl), on hepatic Se, GPx, and the induction of cytochrome P450 1A1. We tested the hypothesis that exposure to PCB 77 produces oxidative stress-related changes in Se concentration and the activity of GPx in the liver by conducting a time-course study using male and female rats, which received a single i.p. injection of PCB 77 (300 μmol/kg). Our results showed that PCB 77 significantly affected the redox status of the liver by reducing the activity of glutathione peroxidase and by decreasing Se levels. In addition, the effect of PCB 77 on the hepatic Se level and GPx activity may be influenced by gender.

We proposed that reduction of the glutathione peroxidase activity resulted from hepatic depletion of Se, which may be a consequence of PCB 77 enhanced transport of Se

to other tissues as well as increased Se excretion. In the next study, we therefore determined the concentration of Se in different tissues, urine and feces of female rats administered a single dose of PCB 77. Similar to previous results, PCB 77 decreased the Se content of the liver as well as the GPx1 activity. This PCB 77 effect may be a result of increased excretion of Se in urine but not in feces. Similar to observations of previous studies on the contrasting regulation of GPx and thioredoxin reductase (TrxR) [204], we found that the TrxR activity was maintained while GPx activity was sacrificed during conditions of inadequate hepatic Se. Although the Se concentrations in tissues were not altered by PCB-77, the relative total Se content of the spleen and muscles was increased compared to the liver, indicating the possible transport of Se from the liver to the spleen and muscles. Further studies on the effect of PCB-77 on the distribution of GPx and TrxR as well as selenoprotein P (SePP), which has a transport or supply function in liver and in extrahepatic organs, will elucidate the extra-hepatic tissue transport hypothesis.

In the third study, we tested the hypothesis that dietary Se supplementation would provide protection against the tumor promoting activities of two PCBs congeners, PCB 77 and a non-coplanar PCB, 2,2',4,4',5,5'-hexaclorobiphenyl (PCB 153), using a 2-stage carcinogenesis model. The female rats were initiated with a single dose of DEN, prior to receiving 4 i.p. injections of corn oil, PCB-77 or PCB-153 (300 μMol/kg body weight, once every two weeks). The rats were fed diets containing different levels of Se (0.02, 0.2, or 2.0 ppm as selenite) during the promotion period. Using placental glutathione *S*-transferase (PGST)-positive as a marker to quantify the altered hepatic foci (AHF), our findings showed that Se supplementation did not diminish the induction of hepatic foci by either PCB 77 or PCB 153. Instead of protection, the number of AHF in the liver and number of foci per cubic centimeter of liver among the PCB 77 treated rats were increased as the Se dietary level increased. Unlike PCB 77, PCB 153 did not show the same Se dose-response effect; nevertheless Se supplementation did not confer protection against foci development. On the other hand, supranutritional Se reduced the mean focal volume. Cell proliferation, measured by the BRDU index, was not inhibited by Se in the liver of the PCB treated groups. Se did not prevent PCB 77 induced decrease of hepatic Se and the associated reduction in GPx activity. In contrast, TrxR activity was not affected by the PCBs treatment or by Se supplementation.

The fourth study aimed at comparing the graphite furnace atomic absorption spectrometry (GFAAS) procedure for analyzing Se in biological samples with established methods, the neutron activation analysis (NAA) and fluorometric methods. The results showed that the measured Se concentration of split samples using GFAAS with Zeeman background correction after closed vessel microwave digestion of samples was lower compared to that using the NAA or the fluorometric methods. Modifications to the GFAAS operating system and refinement of the sample preparation to minimize Se loss will need to be undertaken to improve the detection of Se by this method.

In conclusion, our studies showed that by reducing the antioxidant activity of GPx1, PCB 77 may significantly alter the redox status of the liver. In addition, the effect of PCB 77 on the hepatic selenium level and GPx activity was possibly influenced by gender. The PCB-77 effect on hepatic Se may be a result of increased excretion of Se in urine, but not in feces. PCB 77 and PCB 153 had an effect on GPx1 activity, but not TrxR activity, indicating that TrxR is maintained while GPx1 activity is sacrificed during conditions of PCBs induced hepatic Se depletion. Se supplementation did not ameliorate the PCB 77-induced decrease in hepatic Se and GPx1 activity. The apparent repression of GPx1 activity by PCB 77 was associated with an increased number of foci per liver and increased focal volume ratio despite Se supplementation. In contrast, supranutritional Se reduced the mean volume of the foci, indicating a possible anti-cancer protective effect. Se does not protect against PCBs induced tumor promotion via cell proliferation inhibition and TrxR/thioredoxin antioxidant action. Lastly, the Zeeman-GFAAS method for selenium determination in biological samples together with closed microwave digestion technique was not as reliable as the NAA and the fluorometric methods.

Future Studies

We have shown that PCB 77 affects the hepatic activities of GPx1 and TrxR in a contrasting manner. We also know that PCB 77 depresses GPx1 expression in the liver [317]. It will be interesting to know whether TrxR and SePP expression as well as other selenium binding proteins are affected by PCBs, which could mean that PCBs effect may be directed on the selenoproteins synthesis machinery. A coplanar PCB has been reported to induce a 54-kDa selenium binding protein in the rat liver, which was observed to be similar to a nuclear 56 kDa Se-binding protein that is expressed highly in liver, kidney and liver tumors [506-508]. This 54-kDa selenium binding protein may have a role in the increased excretion of selenium in urine, and therefore, worth looking into.

SePP has 10 selenocysteine residues and synthesized mainly in the liver, hence, it is biologically implicated in the transport and distribution of Se from the liver to other organs [100,102,105,589]. Furthermore, Se deficiency did not reduce the concentration of SePP as strongly as GPx1; and the absence of SePP in SePP deficient mice resulted in decreased Se and selenoenzymes in certain tissues [463]. The latter suggests that impaired transport of hepatic intracellular Se is a loss of function of SePP. Since PCBs appear to increase the relative selenium concentration in the muscles and spleen, investigating the role of SePP in the liver and these organs would elucidate the extra-hepatic transport hypothesis for PCBs induced hepatic depletion.

We have suggested that PCB 77 may be affecting the methylation process; and therefore, may disturb hepatic Se homeostasis. It was noted that Se concentration in urine of mice was high when Se methylation reaction was depressed indicating that the inorganic Se in urine may be contributing to increased Se excretion [143]. The major selenium metabolite in urine is trimethylselenonium ion and recently, another Se metabolite in the form of selenosugar was observed in rats urine [505]. This selenosugar was also detected in the liver. Knowing the particular selenium species in urine that could be affected by PCBs through selenium speciation methods will contribute to understanding the mechanism by which PCBs affect hepatic selenium.

With the increasing interest in using selenium in cancer or tumor prevention, it is important to understand why selenium supplementation increased the development of PCBs-induced preneoplastic lesions and yet decreased the size of the focal lesions. It has

been reported that selenium may be preventive during the tumor progression phase, that is, the nodules do not become hepatocellular carcinomas [237]. A longer duration PCBs or TCDD carcinogenesis study could show whether the preneoplastic lesions will not progress to tumors. Also, categorizing the foci into small, medium and large size lesions and comparing their proportion to the control will verify if indeed the focal size is diminished by selenium.

Lastly, the Zeeman-GFAAS coupled with closed microwave digestion is a safer, cheaper and more accessible alternative to NAA and fluorometric methods in analyzing for selenium; hence, studies on improving its recovery and replicability by modifying some digestion and/or operating conditions would be important.

APPENDIX: ABBREVIATIONS

2-AAF - 2-acetylaminofluorene
AFB1 - aflatoxin B1
AHF - altered hepatic foci
DIO – 5'- iodothyronine deiodinases
DMSe – dimethylselenide, $(CH_3)_2Se$
DMBA - 7,12-dimethylbenz(a)anthracene
GPx - glutathione peroxidases
GSH - glutathione
GSSeSG – selenodiglutathione
GSSG - oxidized glutathione
H_2Se - hydrogen selenide, selenide
MSe, MMSe – monomethylselenol, methylselenol, selenol, $(CH_3)Se^-$
MSC – methylselenocysteine
MsrB - methionine-sulfoxide reductase, selenoprotein R
Se - selenium
SeBP - Se binding or containing proteins
Secys - selenocysteine
SECIS – selenocysteine insertion element
Secys tRNA – selenocysteine transfer RNA
SeMet – selenomethionine
SePP - Selenoprotein P
Sep W - Selenoprotein W
SPS2, Sps2 - selenophosphate-synthetase
PB - phenobarbital
PCBs – polychlorinated biphenyls
PCB 77 - 3,3',4,4'-tetrachlorobiphenyl
PCB 153 - 2,2',4,4',5,5'-hexachlorobiphenyl
p-XSC - 1,4-phenylenebis(methylene) selenocyanate
Trx - thioredoxin
TrxR - thioredoxin reductases
TMSe - trimethylselenonium ion $(CH_3)_3Se^+$

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