

Transcriptional Activation of Stress Genes and Cytotoxicity in Human Liver Carcinoma (HepG₂) Cells Exposed to Pentachlorophenol

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Received: 7 June 2002 / Accepted: 30 September 2002 / Published: 30 September 2002

Abstract: Pentachlorophenol (PCP) is a biocidal chemical with several industrial, agricultural, and domestic applications. There is accumulating evidence indicating that PCP is highly toxic to humans, with major target organs including the lung, liver, kidneys, heart, and brain. Little is known regarding the molecular basis by which PCP induces toxicity, mutagenesis, and carcinogenesis. Therefore, this research was designed to assess the cellular and molecular responses of HepG₂ cells following exposure to PCP. The cytotoxicity experiment yielded a LD₅₀ value of 23.4 ± 9.7 µg PCP/mL upon 48 hrs of exposure, indicating that PCP is acutely toxic. A dose-response relationship was recorded with respect to gene induction. For example, fold inductions of CYP1A1 were 1.0 ± 0.0 , 1.0 ± 0.0 , 1.3 ± 0.5 , 6.3 ± 4.3 , and 22.5 ± 3.5 for 0, 6.2, 12.5, 25, and 50 µg PCP/mL, respectively. Overall, five out of the thirteen recombinant cell lines tested showed inductions to statistically significant levels ($p < 0.05$). At 50 µg PCP/mL, the average fold inductions were 22.5 ± 3.5 , 52.8 ± 2.5 , 8.4 ± 1.9 , 6.16 ± 2.4 , and 12.5 ± 6.8 , for CYP1A1, XRE, HMTIIA, *c-fos*, and GADD153, respectively. These results indicate the potential of PCP to undergo Phase I biotransformation in the liver (CYP1A1, XRE), to cause cell proliferation (*c-fos*), growth arrest and DNA damage (GADD153), and to influence the toxicokinetics of metal ions (HMTIIA). Marginal inductions were recorded for HSP70, CRE, RARE, GADD45, and GRP78. Within the dose range (0-100 µg/mL) tested, no significant inductions ($p < 0.05$) were observed for GSTY_a, NFκBRE, and p53RE.

Keywords: Pentachlorophenol, cytotoxicity, gene expression, HepG₂ cells.

Introduction

Wood is the most prevalent construction material used in the United States. Historically, preserving wood and wood products from deleterious conditions has been a prominent industry in the United States for more than 100 years [1]. It is estimated that more than 700 wood preserving sites exist in the United States and have been identified where wood preservation is or has been conducted or where wood preserving wastes have been identified [1]. Many wood preservation facilities are found in the southern, southeastern and northwestern regions where preferred timber areas have been identified [2]. The most common industrial wood preservatives used in the United States are creosote, chromated copper arsenate, and pentachlorophenol (PCP) [1]. However, PCP is preferred over other wood preservatives because PCP-treated wood retains its natural appearance, has little or no odor, and can be painted as readily as natural wood [3].

PCP is a commercially manufactured organochlorine compound and biocidal chemical with industrial, agricultural, and domestic applications. PCP was first manufactured in 1841, however, it was produced commercially in 1936 [3,4]. It is the third most heavily used pesticide, preceded by the herbicides atrazine and alachlor [5]. The primary use of PCP is to protect timber from fungal rot and wood-boring insects, thus extending the life of wood products such as utility poles, cross arms, fence posts, and similar structures. However, it has also been used in other applications such as leather tanning, cooling-tower algae and fungi control, joint preservation, slime and fungus control in photographic solutions, and in other industrial activities for protection of plants and products from biological degradation [6-8]. Various trade names/synonyms for PCP include: PCP, Penchlorol, Dowicide 7, Dowicide 6, Dowicide EC-7, Durotox, EP 30, Permagard, Permasan, Permatox DP-2, Fungifen, DuraTreet II, Glazd Penta, Woodtreat, PentaReady, PentaWR, Chem-tol, Cryptogil oil, Permite, Priltox, Ortho Triox, Santobrite, Weedone, and Term-I-Trol [7,9].

Much has been reported about the deleterious effects of organochlorine compounds on organ systems. When released into the environment, these chemicals can interfere with the physiological performance of the endocrine, nervous, and reproductive systems. They can also influence sex differentiation in wildlife animals [10-12]. Furthermore, considerable evidence shows that exposure to low levels of organochlorine pesticides can result in physiological stresses and reproductive disruption in humans and other animal species [6,13-17]. PCP's environmental pathways lead to the contamination of soil, water, and food. Soil contamination is the result of runoff from the past use of PCP as herbicide, leachate from treated wood products, spills at industrial facilities and hazardous waste sites [18]. Drinking water, surface water, groundwater, rain, snow, air and aquatic biota are common sources for PCP-contamination in the United States [19].

Significant routes of exposure to PCP include pulmonary absorption of vapors, aerosols, dusts, and absorption via the skin and gastrointestinal tract. Exposure to PCP has the potential to modulate neurological responses; causes impairment to DNA that may lead to cancer; and influences hematological and immunological dysfunctions [15,20]. The major target organs in humans affected

by chronic exposure to PCP include the liver, kidneys, and central nervous system [21]. Acute PCP exposure in humans may induce symptoms of lethargy, tachypnea, tachycardia, intermittent delirium, cerebral edema, and swelling of the myelin sheath [18]. However, under prolonged exposure conditions, adverse reproductive effects in humans are associated with changes in the endocrine gland function and immunological dysfunction [22].

Animal studies show that PCP has the potential to induce tumorigenic [23] and carcinogenic activity [18] in mice. Similarly, infrequent liver tumors, adrenal medulla pheochromocytomas and hemangiomas in mice have been documented [24,25]. Evidence of human mutagenicity and carcinogenesis due to PCP exposure is inadequate, however, based on animal laboratory studies, PCP has unequivocally been established as a carcinogen, and therefore a probable human carcinogen-Group B₂ [24,25].

The literature is limited with scientific information regarding the molecular mechanisms by which PCP induces toxicity, mutagenesis, and carcinogenesis. In order to predict the molecular mechanisms by which PCP exerts its toxic action, we performed the MTT assay to evaluate the cytotoxicity, and the mammalian gene profile (CAT-Tox) assay to assess the transcriptional activation of stress genes in HepG₂ cells exposed to PCP.

Materials and Methods

Chemicals

Pentachlorophenol (C₆Cl₅OH, CAS No. 87-86-5, Lot No. 01530TS), with purity 98.0% was purchased from Aldrich Chem Co., Milwaukee, WI. Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM, Lot No. 109721) was purchased from Life Technologies in Grand Island, New York.

Gene Profile and Cytotoxicity Assays

The CAT-Tox assay was performed for measuring differential gene expression in immortalized human liver cells. The parental cell line (HepG₂), and thirteen different recombinant constructs generated by creating stable transfectants of different mammalian promoter – chloramphenicol acetyltransferase (CAT) gene fusions were obtained from Xenometrix, Inc. (Boulder, CO). Each construct contained a unique stress gene promoter or response element fused to the CAT reporter protein.

In the laboratory, cells were stored in liquid nitrogen until use. They were next thawed by gentle agitation of their containers (vials) for 2 minutes in a water bath at 37°C. After thawing, the content of each vial was transferred to a 75 cm² tissue culture flask, diluted with DMEM supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin, and incubated for 24 hours at 37°C in a 5% CO₂ incubator to allow the cells to grow, and form a monolayer in the flask. Cells grown to 80-95% confluence were washed with phosphate buffer saline (PBS), trypsinized with 10 mL of 0.25%

(w/v) trypsin-0.03% (w/v) EDTA, diluted, counted, and seeded (5×10^5 cells/well) in two sets of 96-well microtiter tissue culture plates.

Seeded plates were incubated for 24 hrs at 37°C in a 5% CO₂ incubator, followed by a replacement of the old medium by a fresh one containing the appropriate amount of the test chemical: 0, 6.2, 12.5, 25, 50, and 100 µg/mL PCP in 1% DMSO. For quality assurance/quality control purposes, positive control plates were also be made using known inducers including 3-methyl cholanthrene (3-MC-10 µM) for CYP1A1, CRE, GADD45, p53RE, and XRE; methyl methane sulphonate (MMS-100 µg/mL) for GSTY_a, HMTIIA, *c-fos*, HSP70, NFκBRE, GADD153, and GRP78; and all-*trans* retinoic acid (RA-10 µM) for RARE. All chemical exposures involved the use of polypropylene 96-well microtiter plates for the purpose of chemical dilutions. A specific, constant volume (20 µL) was transferred from each well of the chemical dilution plate to the plate containing the cells to give each cell line five chemical doses and a zero control dose, each in triplicate.

Following chemical exposure, the cells were be re-incubated for 48 hrs at 37°C, and 5% CO₂. After the incubation period, the total protein was measured by the Bradford method, at 600 nm using a microtiter plate reader (Bio-Tek Instruments Inc.). A standard sandwich ELISA was performed and in the final step, horse radish peroxidase catalyzed a color change reaction that was measured at 405 nm using a Bio-Tek microplate reader [26,27].

The parental HepG₂ cell line was dosed in the same manner as the recombinant cell lines, and was used to perform the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based cell viability assay using a microtiter plate reader (Bio-Tek Instruments Inc.) with the wavelength set at 550 nm [28].

Statistical Analysis

The transcriptional fold inductions for each recombinant cell line at each PCP concentration were calculated using the CAT-Tox computer software based on the optical density readings at 600 and 405 nm. The software also converted the 550-nm readings to cell viability percentages. Standard deviations were determined, and the *Student's t-test* values were computed to determine if there were significant differences in cell viability and gene induction in treated cells compared to the control cells. Graphs were made to illustrate the dose-response relationship with respect to cytotoxicity and gene expression.

Results

The effects of PCP (100 µg/mL in 1% DMSO) on parental HepG₂ cells are shown in Figure 1. PCP cytotoxicity to HepG₂ cells was assessed using the MTT-assay for cell viability. HepG₂ cells were exposed to serial concentrations of PCP (0-100 µg/mL) for 48 h. Toxic end-points estimated, included the no observable adverse effect level (NOAEL), the lowest observable adverse effect level (LOAEL), and the median lethal concentration (LC₅₀). A classical concentration-response pattern was demonstrated for the 48 h exposure to PCP. PCP concentrations of 6.2 ± 2.5 , 12.5 ± 4.8 , and 23 ± 5.6

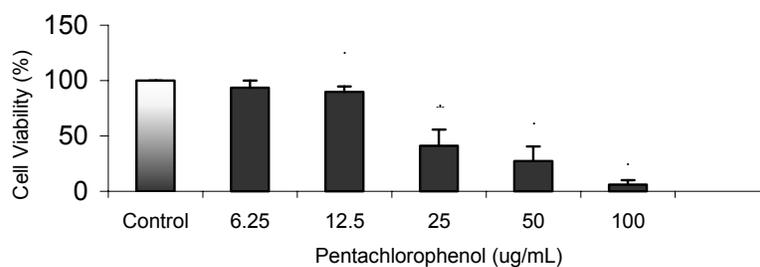


Figure 1. Toxicity of pentachlorophenol to human liver carcinoma cells (HepG₂): HepG₂ cells were treated with serial dilutions (0-100 µg/mL) of PCP. Cell viability was measured by MTT assay as indicated in the methodology section. Absorbance readings taken from survival cells were converted to percentage cell viability using the CAT-Tox software. Bars are means ± SDs, n=3 with 8 replications per concentration. All values are significantly different ($p \leq 0.05$) from control (0 µg/mL PCP), except for 62.5 µg/mL.

µg/mL were recorded for NOAEL, LOAEL, LC₅₀, respectively, indicating that PCP is acutely toxic to HepG₂ cells.

Transcriptional gene responsiveness of HepG₂ cells following 48 h exposure to PCP was assessed by performing the CAT-Tox assay. The CAT-Tox assay consists of 13 stress promoter-chloramphenicol acetyl transferase (CAT) fusion constructs generated from the human hepatoma cell line, HepG₂ (Table 1). Overall data revealed that five (CYP1A1, XRE, HMTIIA, *c-fos*, and GADD153) out of the 13 constructs showed statistically significant ($p < 0.05$) induction/activation trends associated with 48-h exposure to serial concentrations of pentachlorophenol (0-100 µg/mL). All fold inductions patterns were observed as concentration-dependent occurrences.

Table 1. Stress gene promoter/response element—CAT fusion constructs and their biologic functions

<i>Phase I Metabolism</i> <i>PAH Induction</i>	<i>Heavy Metal</i> <i>Sequestration</i>	<i>Growth arrest</i> <i>DNA-Damage</i>
CYP1A1	HMTIIA	GADD153/GADD45
<i>Phase II Metabolism</i> <i>PAH Induction</i>	<i>Protein Chaperone</i> <i>Heat Induction</i>	<i>Binding Site for</i> <i>Retinoic Acid</i>
GST YA	HSP70	RARE
<i>AH Binding</i> <i>Sequence</i>	<i>cAMP Reporter</i> <i>Element</i>	<i>AP-1 Factor Component</i>
XRE	CRE	FOS
<i>ER Protein</i> <i>Chaperone</i>	<i>Oxidative Stress</i> <i>Mitogen Induction</i>	<i>Programmed Cell Death/</i> <i>Apoptosis Cell Cycle Arrest</i>
GRP78	NFκBRE	p53RE

The CYP1A1 gene promoter fused to the CAT protein was treated with serial dilutions of pentachlorophenol (0-100 $\mu\text{g/mL}$), as shown in Figure 2. An increase in CYP1A1 fold induction was shown to be concentration-dependent over the range of 0 to 50 $\mu\text{g/mL}$ PCP. A significant ($p < 0.05$) CYP1A1 fold induction of 22.5 ± 3.5 was observed at 50 μg PCP /mL. A decline in CYP1A1 fold induction was also observed at 100 μg PCP/mL, probably due to cell death at higher doses of PCP exposure. A fold induction of 52.8 ± 2.5 was observed in the xenobiotic response element (XRE) at 50 μg PCP/mL as shown in Figure 3. Although this fold induction was statistically significant ($p < 0.05$), no other inductions were statistically different from the control.

The HMTIIA gene promoter fused to the CAT protein was treated with serial dilutions of pentachlorophenol (0-100 $\mu\text{g/mL}$). An increase in HMTIIA fold induction was shown to be concentration-dependent over the range of 0 to 50 $\mu\text{g/mL}$ PCP. A significant ($p < 0.05$) HMTIIA fold induction of 8.4 ± 1.9 was observed at 50 μg PCP/mL as shown in Figure 4. A slight fold induction was seen at 12.5 μg PCP /mL, however, it was not statistically significant ($p > 0.05$).

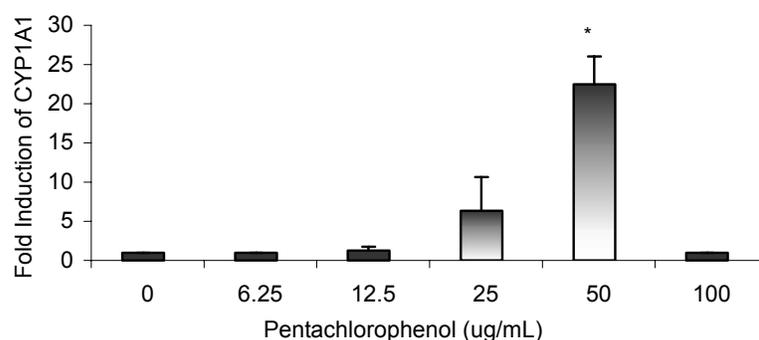


Figure 2. Fold induction of CYP1A1 in HepG₂ cells exposed to pentachlorophenol: Recombinant HepG₂ transfected with CYP1A1 gene promoter fused to the CAT protein were treated with serial dilutions of PCP (0-100 $\mu\text{g/mL}$). CYP1A1 induction was based on the expression of CAT-protein using a sandwich ELISA, as indicated in the methodology section. Bars are means \pm SDs, $n=3$. *Significantly different from control (0 $\mu\text{g/mL}$ PCP), $p \leq 0.05$.

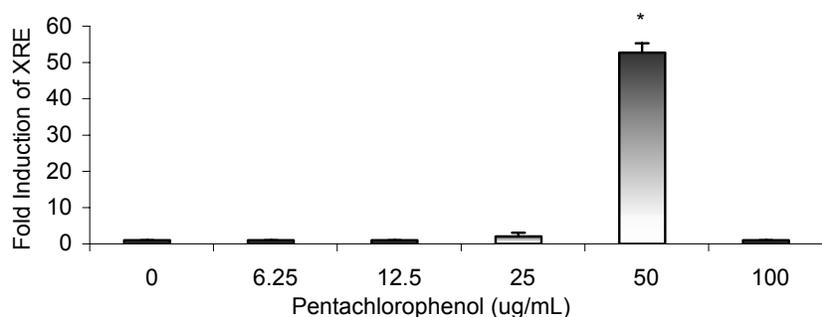


Figure 3. Fold induction of XRE in HepG₂ cells exposed to pentachlorophenol: Recombinant HepG₂ transfected with a XRE gene promoter fused to the CAT protein were treated with serial dilutions of PCP (0-100 $\mu\text{g/mL}$). XRE induction was based on the expression of the CAT-protein using a sandwich ELISA, as indicated in the methodology section. Bars are means \pm SDs, $n=3$. *Significantly different from control (0 $\mu\text{g/mL}$ PCP), $p \leq 0.05$.

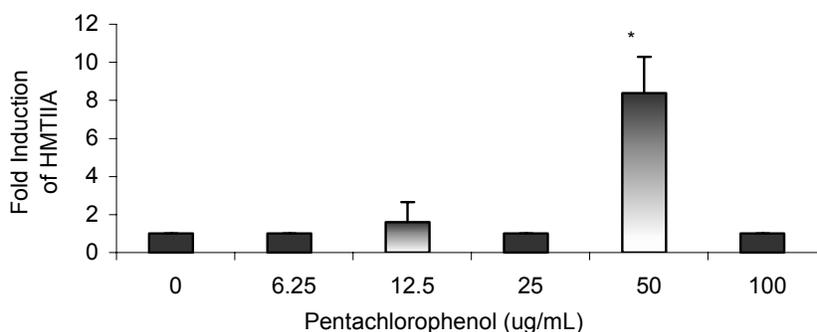


Figure 4. Fold induction of HMTIIA in HepG₂ cells exposed to pentachlorophenol: Recombinant HepG₂ cells transfected with a HMTIIA gene promoter fused to the CAT protein were treated with serial dilutions of PCP (0-100 µg/mL). HMTIIA was based on the expression of the CAT-protein using a sandwich ELISA, and indicated in the methodology section. Bars are means \pm SDs, n=3. *Significantly different from control (0 µg/mL PCP), $p \leq 0.05$.

The *c-fos* gene promoter fused to the CAT protein was treated with serial dilutions of pentachlorophenol (0-100 µg/mL). *c-fos* showed a statistically significant ($p < 0.05$) increase in gene induction (6.16 ± 2.4) at 50 µg PCP/mL (Figure 5). No other significant inductions were observed at other PCP concentrations.

The GADD153 gene promoter fused to the CAT protein was treated with serial dilutions of pentachlorophenol (0-100 µg/mL). A significant ($p < 0.05$) increase in GADD153 gene induction 12.5 ± 6.8 was observed at 50 µg PCP /mL as shown in Figure 6. Marginal inductions were recorded for HSP70, CRE, RARE, GADD45, and GRP78. However, these inductions were not statistically significant ($p > 0.05$) probably due to their relatively high standard deviations associated with their mean values (Figure 7). No significant inductions ($p > 0.05$) were observed for GSTY α , NF κ BRE, and p53RE.

Discussion

Cytotoxicity Assay

In the present study, PCP was acutely toxic to human liver carcinoma (HepG₂) cells (Figure 1). The MTT assay was employed to assess cell viability. The sensitivity of the tetrazolium salt reduction (MTT) assay was based on the ability of succinate-dehydrogenase, a mitochondrial enzyme, to convert MTT to a water-insoluble formazan dye in viable cells. This mitochondrial activity was demonstrated when exposure to PCP significantly reduced the viability of HepG₂ cells ($LC_{50} = 23.4 \pm 5.6$ µg/mL).

Toxicity to HepG₂ cells is often an elaboration of concentration-dependent occurrences. In the present study, PCP's toxic potency to HepG₂ was concentration-dependent, indicating that increased levels of PCP caused increased toxicity to HepG₂ cells. Our results are consistent with a previous study that reported a strong concentration-response relationship with respect to the cytotoxic effects of

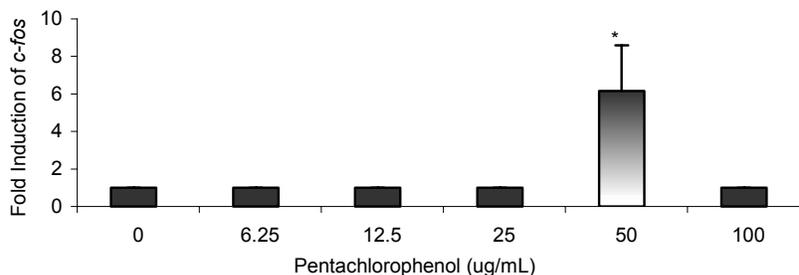


Figure 5. Fold induction of *c-fos* in HepG₂ cells exposed to pentachlorophenol: Recombinant HepG₂ transfected with a *c-fos* gene promoter fused to the CAT protein were treated with serial dilutions of pentachlorophenol (0-100 µg/mL). *c-fos* induction was based on the expression of the CAT-protein using a sandwich ELISA, as indicated in the methodology section. Bars are means \pm SDs, n=3. *Significantly different from control (0 µg/mL PCP), $p \leq 0.05$.

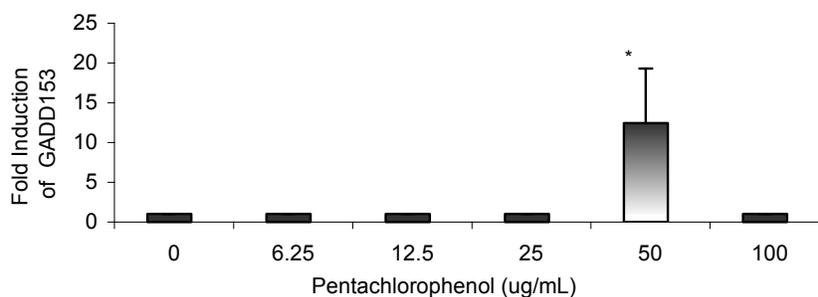


Figure 6. Fold induction of GADD153 in HepG₂ cells exposed to pentachlorophenol: Recombinant HepG₂ cells transfected with a GADD153 gene promoter fused to the CAT protein were treated with serial dilutions of pentachlorophenol (0-100 µg/mL). GADD153 induction was based on the expression of CAT-protein using a sandwich ELISA, as indicated in the methodology section. Bars are means \pm SDs, n=3. *Significantly different from control (0 µg/mL PCP), $p \leq 0.05$.

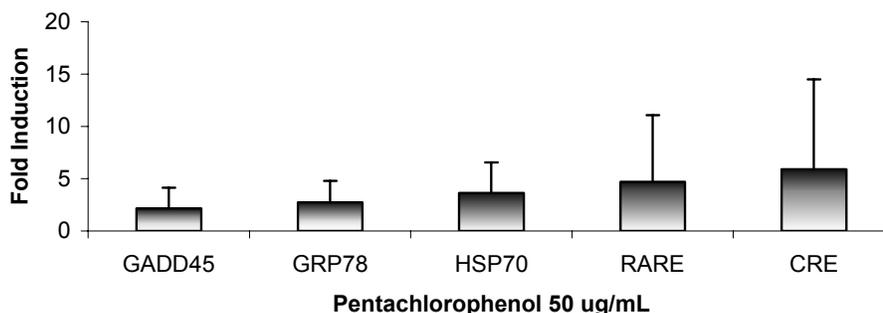


Figure 7. Marginal inductions of GADD45, GRP78, HSP70, CRE, and RARE in HepG₂ cells exposed of 50 µg/mL of pentachlorophenol: Recombinant HepG₂ cells transfected with a GADD45, GRP78, HSP70, CRE, and RARE gene promoters fused to the CAT protein were treated with serial dilutions of PCP (0-100 µg/mL). GADD45, GRP78, HSP70, CRE, and RARE inductions were based on the expression of the CAT-protein using a sandwich ELISA, as indicated in the methodology section. Bars are means \pm SDs, n=3. Values are not significantly different from control (0 µg/mL PCP), $p > 0.05$. trinitrotoluene to HepG₂ cells [30]. In the trinitrotoluene study, the LC₅₀ was around 100 µg/mL,

indicating that PCP is more toxic to HepG₂ cells as compared to TNT. Another study reported that isomers of endosulfan (alpha and ss) caused concentration-dependent genotoxic effects in HepG₂ cells [31]. Increased concentrations of alpha- and ss-endosulfan caused an increase in sister chromatid exchanges in HepG₂ cells upon 48 h of exposure, as well as DNA strand breaks within 1 h of exposure.

Gene Profile Assay

The current study confirms previous works showing that various chemical compounds have the potential to up-regulate stress genes in human liver carcinoma (HepG₂) cells [26,29,30]. Exposure of HepG₂ cells to PCP revealed a significant induction of a number of stress genes including CYP1A1, XRE, HMTIIA, *c-fos*, and GADD153. Further, several studies report that metal and nonmetal compounds modulate transcriptional activation of stress genes in the human hepatoma cell line, HepG₂ [26,29,30]. Moreover, several reports in the literature have addressed the potential of organochlorine pesticides to initiate reporter gene transcription from estrogen response elements in DNA [32].

CYP1A1 – cytochrome P450 1A1 is the predominant microsomal enzyme that is responsible for Phase I metabolic activities. Subsequently, the binding of CYP1A1 substrates to the aryl hydrocarbon (Ah) receptor forms the Ah receptor-substrate complex. Further, CYP1A1 is induced when the Ah receptor-substrate complex binds to the xenobiotic response element (XRE). Induction of the CYP1A1 gene in PCP-treated HepG₂ cells implicates the involvement of Phase I metabolic machinery (Figure 1). The potential of pentachlorophenol to up-regulate CYP1A1 is linked to the binding affinity of pentachlorophenol to the aryl hydrogen receptor (AhR). The AhR is recognized as an intracellular mediator of the xenobiotic signaling pathway [32]. The AhR is a basic helix-loop-helix protein belonging to the Per-Arnt-Sim family of transcription factors. Upon exposure, PAHs and chlorinated compounds bind to the AhR which in turn modulates the activity of Phase I and Phase II biotransformation enzymes, thus increasing them in concentration.

When xenobiotic compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene (3-MC) bind to the AhR, the ligand-receptor complex is translocated from the cytosol to the nucleus where it dimerizes with the AhR nuclear protein [33,34]. In a previous study, it was found that exposure of HepG₂ cells to phorbol 12-myristate 13-acetate (PMA) dramatically enhanced transcriptional activation of the CYP1A1 gene [35]. In the present study, the CYP1A1 gene protein in HepG₂ cells was up-regulated by PCP. These findings suggest that CYP1A1 gene amplification is mediated through the xenobiotic response element–XRE [36,37] and provide direct evidence that the Phase I biotransformation pathway is operational in PCP-treated HepG₂ cells.

XRE – xenobiotic response element is identified as the binding site for the Ah receptor-substrate complex. XRE plays a central role in metabolism that involves Phase I and Phase II biotransformation enzymes and growth factors. Consistent with the concentration-dependent induction of the CYP1A1 gene protein, the induction of XRE at 50 µg/mL is up-regulated as a result of PCP exposure, with a maximum activation at 50 µg/mL (Figure 3). It has been established that the XRE sequence is a

regulatory sequence that functions as the AhR-mediated xenobiotic signal transduction system [33]. The XRE consensus sequence is located in the within the 5'-flanking region of the CYP1A1 gene and stimulates CYP1A1 protein synthesis [33,38]. Accordingly, the amplified expressions of the CYP1A1 gene and XRE in PCP-treated HepG₂ cells suggests that the expression is a result of physical interaction between AhR complex and binding XRE promoter region of the CYP1A1 gene. Mammalian liver cells contain a microsomal pool of cytochromes P450 that play an essential role in biotransformation reactions, therefore increasing the water solubility of endogenous and exogenous compounds. This detoxification process is intimately linked with the activities of biotransformation enzymes. The biological activities of ligands such as polychlorinated dibenzo-*p*-dioxins, polycyclic aromatic hydrocarbons (PAHs), benzimidazoles, and bioflavonoids are mediated through the aryl hydrogen receptor (AhR).

c-fos is recognized as an immediate early gene (IEG) and has been identified as a transcription factor that responds to DNA-damage. *c-fos* is a component of the activator protein-1 (AP-1) complex and can also be amplified by oxidative stress and phorbol ester compounds. The *c-fos* gene protein has been implicated in the cell proliferation machinery. *c-fos* expression is part of a mitogenic response that is required for cell proliferation. The up-regulation of this transcription factor is stimulated by DNA-damaging agents [39]. Further, the induction of the *c-fos* gene can be modulated via the mitogen-activated protein kinase pathway. In the present study, a dose-response relationship was observed with regard to the effect of pentachlorophenol on the transcriptional activation of the *c-fos* gene promoter (Figure 5). Our data suggest that pentachlorophenol is a DNA-damaging agent. Moreover, the induction of the *c-fos* gene involves both transcriptional and post-transcriptional machinery. Once stimulated, *c-fos* conjoins with *c-jun*, a transcription factor of the Jun family, and forms the heterodimeric complex, activator protein-1 (AP-1). Although the *c-fos* protein is identified as an IEG and a component of the AP-1 consensus, the AP-1 complex transactivates a cascade of other late genes [40]. *c-fos* as an IEG response may therefore be involved in a pentachlorophenol-induced toxic cascade or in the deleterious effects of pentachlorophenol.

Metallothioneins are a family of low molecular weight, cysteine-rich proteins that function in metal detoxification and trace metal homeostatic mechanisms. The up-regulation of the HMTIIA promoter is ideally elicited by toxic metals such as cadmium, zinc, copper, silver and arsenic [26,41]. Accordingly, it has been espoused that metallothioneins act as intracellular storage and concentration sites for heavy metals [30]. However, oxidative stress has been shown to initiate increased levels of metallothionein which in turn act as a scavenger of reactive oxygen species. Consequently, it has been suggested that metallothionein I and II may play a role in the protection against oxidative stress [42]. In the present study, the HMTII_A gene is amplified in PCP-treated HepG₂ and shows a concentration-dependent occurrence (Figure 4). Pentachlorophenol is not a metal, and therefore, induction of the HMTIIA gene promoter is believed to be an indirect consequence from oxidative stress. This finding suggests that there is a possible pool of non-metal transcription factors that elicit the up-regulation of

the HMTII_A gene upon PCP exposure in HepG₂ cells. Consequently, it has been suggested that metallothionein I and II may play a role in the protection against oxidative stress [42]. It has been demonstrated in previous studies that the HMTIIA promoter contains a steroid-like response element (glucocorticoid) and is activated by steroid hormones and their analogs [41].

GADD153 – 153 kDa growth arrest and DNA damage protein is exclusively responsive to DNA damage. Moreover, GADD153 contains an AP-1 site within its promoter region that is unresponsive to phorbol ester compounds. Our studies demonstrate that PCP toxicity causes DNA damage to HepG₂ cells following 48 hr of exposure (Figure 6). Accordingly, PCP caused strong induction folds of GADD153 (Fig. 6). Up-regulation of GADD153 and GADD45 implicates the potential of PCP to cause changes in DNA sequence and conformational changes in the helical structure [30]. Although a marginal induction of GADD45 was co-expressed (Figure 7), these results indicate that PCP is causing DNA damage.

Marginal inductions were reported for the 70-kDa heat shock protein (HSP70), cAMP response element (CRE), retinoic acid response element (RARE), 45-kDa growth arrest DNA damage 45 (GADD45), and 78-kDa glucose regulated protein (GRP78) promoters (Figure 7). The HSP70 gene is a molecular chaperone that is part of a protein family that is involved in protein folding, translocation, and refolding of intermediates and proteases, while ensuring the efficient degradation of damaged and short-lived proteins [43]. Unfolded proteins elicit increased levels of molecular chaperones and enzymes involved in protein folding in the endoplasmic reticulum (ER) [44]. Under stressful conditions, the accumulation of unfolded proteins in the ER leads to the induction of transcription of genes that encode molecular chaperones and folding enzymes [45]. In addition, our results show as the dose of PCP-treated HepG₂ increases, a more amplified expression of HSP70 is observed with a maximum at 50 µg PCP/mL. Although the fold induction of the HSP70 gene is observed as marginal, these results may be directly linked to the phenomenon in which the HSP70 gene enhances a cell-signaling cascade that initiates protein repair [43]. The role of GRP78 has been implicated in coordination of endoplasmic reticular processing. The GRP78 binding protein is a homologue of cytoplasmic HSP70. Our results demonstrate that the GRP78 fold induction highly correlates with the marginal induction of the HSP70 promoter. Although the GRP78 promoter is marginally induced, overexpression of this protein is closely linked with proteins that are misfolded because of mutagenesis and other compromising conditions [46].

The RARE promoter – retinoic acid response element, has been implicated as a receptor for the RAR β complex, an intracellular receptor. The RAR β structure is responsive to steroid-like compounds. This binding mechanism suggests that the RARE promoter is capable of activating the promoters and transcription of downstream genes [27]. Further, retinoids and their analogs have been implicated in biological activities such as growth and differentiation [47]. These compounds are fundamental to normal embryonic development while providing essential differentiation framework. Moreover, exposure to retinoids has resulted in compromised characteristic development of the

craniofacial, facial bone, thymic, and CNS structures [48]. In our study, the marginal induction of the RARE promoter is indicative of the potential for adverse developmental, growth, and differentiation effects caused by PCP.

The GSTY α gene is closely associated to Phase II biotransformation enzymatic activities. However, in the present study, no significant inductions were observed for the GSTY α gene. The functional p53RE, tumor suppressor gene, plays a dual role in response to DNA damage. Initially, G1 and G2 phases of the cell cycle are interrupted by a p53 signal to allow DNA repair to take place. When DNA damage is irreparable, the p53 protein induces the apoptosis pathway. In our study, no significant inductions were observed for the p53 response element promoter. The lack of p53 induction may be due to the potential mitogenic effect of PCP at lower levels of exposure.

Conclusions

Acute exposure to pentachlorophenol significantly reduces the viability of human liver carcinoma (HepG₂) cells; the LC₅₀ was computed to be about 25 μ g/mL PCP; indicating that PCP is acutely toxic to HepG₂ cells. The gene profile (CAT-Tox) assay provided valuable information on the molecular mechanisms of PCP-induced toxicity in HepG₂ cells. PCP involvement in the Phase I bio transformation pathway is characterized by significant inductions of cytochrome P450 1A1 and XRE.

Our findings also demonstrate that under certain conditions that PCP is genotoxic and subsequently can damage DNA by altering DNA sequence. Genotoxicity of PCP was also evidenced by significant inductions of GADD153 (growth arrest and DNA damage) and *c-fos* (DNA repair response and cell proliferation).

Although PCP is not a heavy metal, it has the potential to indirectly influence the induction of HMTIIA, probably as a consequence of oxidative stress; thereby influencing the toxicokinetics of metals. PCP has the ability to cause proteotoxic effects, as characterized by marginal inductions of HSP70 and GRP78 in PCP-treated HepG₂ cells.

Acknowledgements. This research was financially supported by a grant from the U. S. Department of Education through Title III Graduate Education Grant No. P031B990006-01 to Jackson State University. We thank Dr. Abul Mohamed, Dean of the School of Science and Technology, for his technical support on this research project.

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