Long-term p-nitrophenol exposure can disturb liver metabolic cytochrome P450 genes together with aryl hydrocarbon receptor in Japanese quail.
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Received for publication, December 10, 2014; accepted, July 6, 2015

Abstract

\( P\)-Nitrophenol is a major metabolite of some organophosphorus compounds. It is considered to be one of nitrophenol derivatives of diesel exhaust particles that induce substantial hazards impacts on human and animal health. \( P\)-Nitrophenol (PNP) is a persistent organic pollutant. Consequently, bioaccumulation of PNP potentiates toxicity. The objectives of the current study were to assess the potential hepatic toxicity and pathway associated with long-term exposure to PNP. Japanese quails were orally administered different doses of PNP for 75 days. Liver and plasma samples were collected at days 45 (45D), days 60 (60D) and days 75 (75D). Liver histological changes and plasma corticosterone levels were assessed. Basal mRNA level of cytochromes \( P450 \) (CYP 450) (CYP1A4, 1A5, 1B1), heme oxygenase (HO-1), and aryl hydrocarbon receptor 1 (AhR1), from the liver of exposed birds and primary hepatocytes cultured for 24 hr with PNP, were analyzed using quantitative real-time PCR. The results revealed various histopathological changes in the liver, such as lymphocytes aggregation and hepatocytes degeneration. Significant increases in corticosterone levels were reported. After 60-days of \textit{in vivo} exposure, the birds exhibited an overexpression in the liver CYP1A4, 1B1, AhR1, and HO-1. Furthermore, with continuous PNP administration, an overall downregulation of the tested genes was observed. \textit{In vitro}, although a significant overexpression of CYP1A4, 1B1, and HO-1 was observed, CYP1A5 was downregulated. In conclusion, PNP can interfere with the liver CYP 450 enzymes and modulate HO-1 expression in the \textit{in vitro} and \textit{in vivo} experiments. Hence, it could have serious deleterious effects on humans, livestock, and wild animals.
PNP affects liver CYP450 enzymes and AhR1

Key Words: Aryl hydrocarbon receptor, Cytochrome P450, Nitrophenol, Quail

Introduction

P-Nitrophenol (PNP) is a major metabolite of some organophosphorus (OPs) insecticides such as parathion (O, O-Diethyl p nitrophenylphosphorothioate), fenitrothion (O,O-Dimethyl O-3-methyl-4-nitrophenylphosphorothioate), and methyl parathion (O,O-Dimethyl p-nitrophenylphosphorothioate). Furthermore, PNP is considered not only one of nitrophenol derivatives of diesel exhaust particles (DEPs), but also an intermediate chemical substance used in the manufacture of some drugs, fungicides, and dyes. PNP is commonly found in water, soil, and air from agriculture and industry manufacturing. PNP is a persistent organic pollutant and the illegal use of OPs insecticides increase PNP bioaccumulation. Therefore, OPs insecticides abuse increases humans, livestock, and wild animals’ exposure to PNP toxicity, which exerts a variety of deleterious health impacts with severe environmental pollution. PNP has an adverse impact on some fundamental biological processes such as endocrine system, both male and female reproductive systems, and blood circulation.

Japanese quail (Coturnix japonica) is an interesting domesticated economic bird for commercial meat and egg production. Besides, the Japanese quail is considered an ideal biological and experimental model due to its fast development. Moreover, quail is an accepted model for assessing both acute and chronic effects of pesticides and other chemicals. Quails are vulnerable to OPs and their metabolites toxicity because they have been intensively used for pests control in veterinary farms in different formulations as contact, systemic, and fumigant formulas. Furthermore, OPs cause a potential threat to human health due to the presence of their residues in animals' products. Therefore, quails’ exposure to OPs compounds might cause adverse health impacts and economic losses.

PNP is considered to be one of endocrine disrupting compounds. In this vein, a plethora of previous researches on PNP mainly focused on the reproductive system and ignored the other systems, although there are ample evidences that clarify the existence of a massive amount of “crosstalk” between the endocrine and other organs. This dynamic connection gives the potential to disrupt the development and function of a myriad of organs such as liver, which is responsible for PNP metabolism via CYP 450 enzymes. For instance, a radiolabeled PNP was detected in rats’ livers after a single dermal exposure to radiolabeled methyl parathion. Meanwhile, CYP 450 enzymes levels in chicken and quail livers is not high. As a result, these birds have lower catalytic activity. To sum up, both the unregulated accumulation of PNP and moderate rate of clearance potentiate PNP toxicity in quails.

A previous published study revealed that PNP causes endocrine disruptions in male hormones. It is expected that the hormonal metabolism disruption was caused by liver dysfunction. Despite the potential significant toxic effects, basic data on the toxicity of PNP are very rare. There is a lack of knowledge about the extent to which a long-term low dose (chronic) of PNP exposure affects the liver health condition. In this vein, the objectives of the current study were to assess the potential hepatic toxicity associated with chronic PNP exposure and the possible pathway. The present study goes some way towards addressing how PNP affects liver health condition through modulation of CYP 450, AhR1, and HO-1 expression levels using Japanese quails in vivo using primary hepatocyte culture system. Therefore, the present study was designed to identify the underlying mechanisms
related to potential hepatic problems associated with PNP since the molecular mechanisms of PNP toxicity are not fully understood. Furthermore, the general body stress condition was evaluated through corticosterone level assessment. Moreover, determining the toxicity of exposure to PNP is essential for risk evaluation and provides the necessary precautions.

2. Materials and methods

2.1. Chemicals

P-Nitrophenol crystals (4-nitrophenol; PNP, CAS No. 100–02–7, C₆H₅NO₃, >99.9% purity, molecular weight 139.11), glucagon, collagenase, trypsin inhibitor, insulin, amino acids, penicillin-streptomycin, and basal medium eagle (Sigma-Aldrich Co. Louis St., MO, USA) were used in this experiment.

2.2. Birds and experimental design

Male Japanese quails (aged 4 weeks and weighed 80–90 g) were housed in metal cages in a controlled environment (lights on, 0500–1900 hr, temperature, 24 ± 2°C, humidity, 50 ± 10%, and air exchanged 20 times/hr). Birds were provided with food (Kanematsu quail diet; Kanematsu Agri-tech Co. Ltd, Ibaraki, Japan) and water ad libitum. This study was conducted in accordance with the guideline principles in the Use of Animals in Committee of Tokyo University of Agriculture and Technology. The protocol was approved by the Committee of the Ethics of animal experiments of Tokyo University of Agriculture and Technology.

Quails were randomly divided into four groups (n = 25–26 for each) and assigned to the following treatments: i) control group (phosphate-buffered saline), ii) PNP 0.01 mg/kg b.w. (low dose), iii) PNP 0.1 mg/kg b.w. (mid dose), and iv) PNP 1.0 mg/kg b.w. (high dose). The PNP was dissolved in 0.01 M phosphate-buffered saline (PBS; pH 7.4) and administered to quails daily for 75 days (75D) using plastic stomach tube. The doses were decided depending on the pharmacokinetics of PNP and previous studies on rats.

2.3. Blood and liver samples collection

Five quails per group were weighed and euthanized by decapitation at days 45 (45D), days 60 (60D) and days 75 (75D) post treatment. Animals were treated humanely with regard to alleviating birds suffering. Blood samples were collected in heparinized plastic tubes and centrifuged at 1700 × g for 15 min at 4°C. Then, plasma was separated and stored at −20°C until the corticosterone assay was conducted. The liver was rapidly excised, trimmed of connective tissue, and washed free of blood with 0.9% NaCl solution. Afterward, they were blotted over a piece of filter paper and weighed. A part of the liver from the same lobe was fixed for 24 hr in 4% paraformaldehyde in PBS pH 7.4 (Sigma-Aldrich Chemical). Then, it was kept in 70% ethanol for histopathological examination. The other part was snap frozen and stored at −80°C until used for genes expressions.

2.4. Corticosterone measurement

Plasma concentration of corticosterone was measured by double-antibody radioimmunoassay (RIA) system using 125I-labeled radioligands as described previously. The intra- and inter-assay coefficients of variation were 8.5% and 11.4%.

2.5. Histological examinations

The fixed livers samples were dehydrated through a series of ethanol-graded concentrations, clarified in xylene, embedded in paraffin, and sectioned at 4 μm. The liver sections were stained with hematoxylin and eosin for detection of histological alterations.

2.6. Primary culture of quail hepatocytes

Quail hepatocytes were established in a monolayer culture as described previously. In brief, the quail hepatocytes were isolated from 15 week-old male quails. Hepatocytes collection was
performed under intravenous injection of 5% sodium pentobarbital 0.5 ml/kg body weight. Hepatocytes collection was performed by the in situ perfusion method on two step perfusion at a rate of 12 ml/min. Firstly, liver was perfused with sterile Hank’s balanced salt solution (HBSS) containing 0.19 g/l ethylene glycol tetraacetic acid (EGTA) and 2.38 g/l 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) at 37°C for 5–10 min. After that, perfusion with HBSS containing 0.05% collagenase type I was performed. The liver was carefully removed and placed in a petri dish containing basal medium Eagle. The liver’s capsule was trimmed away, and the hepatocytes were detached by brushing the liver using a plastic comb, then filtered using cotton mesh to remove the clumped cells. The suspension of hepatocytes was incubated in a water bath at 37°C for 10 min, filtered in stainless steel mesh, centrifuged at 450 for 5 min, and filtered through different gradients of cell filters (100, 80, and 40 μM). The supernatant was aspirated, and the sediment was resuspended in the media and recentrifuged several times. Hepatocytes with more than 90% viability (verified by trypan blue exclusion test) were used for subsequent plating. The hepatocytes were plated in 96-well culture plates (0.5 × 10^4 cells/well collagen coated dish) containing 200 μl of cell culture basal medium Eagle supplemented with essential amino acids and containing glucose 3.5 g/l, NaHCO_3 2.2 g/l, penicillin 75 U/ml, streptomycin 75 U/ml, insulin 1 μg/ml, glucagon 1 μg/ml, and quail serum 0.5%. The cultured plates incubated for 24 hr at 37°C in a humidified incubator with 5% CO_2 in the gas phase. After 24 hr incubation, the cultured plates were reincubated for 1 hr in serum free media containing 1 μg/ml insulin, 0.12 μg/ml trypsin inhibitor, and 1 μg/ml glucagon. It was followed by 24 hr incubation in the presence of various concentrations of PNP (1.0, 10.0, and 100.0 μM). Finally, the cultured plates were rinsed two times with PBS, dissociated, and collected for mRNA expression. All analyses were performed on two independent hepatocytes cultured preparations. Concerning the cell viability of cultured hepatocytes, it was measured in three independent hepatocytes cultured preparations using a cell-counting kit-8 (Dojindo Molecular Technologies, Minato-ku, Tokyo, Japan) at zero time and after 24 hr of PNP treatment (0, 10, and 100 nM, 1.0, 10.0, and 100.0 μM). Untreated wells were considered as a positive control, while 99% ethanol treated wells were used as a negative control. Working solution of tetrazolium salt was reduced by dehydrogenase activities in cells to give a yellow formazan dye. It was measured using a colorimetric microplate reader (BIO-RAD) with an emission wavelength of 450 nm. The obtained values were normalized with the control positive values.

2.7. Quantitative real-time PCR

To evaluate the changes in expression levels of CYP 450, AhR1, and HO-1 in vivo and in vitro, total RNA was extracted from cultured hepatocytes and liver tissues from in vivo experiment using trizol reagent (Invitrogen, San Diego, CA, USA). The concentration and purity of the isolated total RNA were determined spectrophotometrically using a Nanodrop lite (Thermo Fisher Scientific Inc. Wyman St., Waltham, MA, USA). Complementary DNA (cDNA) was synthesized using PrimeScript reverse transcriptase (TaKaRa Bio Inc., Otsu, Shiga, Japan) according to the manufacturer’s instructions. PCR reactions were performed using SYBR Premix Ex Taq™ (TaKaRa Bio). Oligonucleotide primers were selected using web-based Primer 3 software and previous papers^{22,55} (Table 1). The relative expression level of each target mRNA was determined using 2^ΔΔCT method^{32}. β-actin was used as the endogenous control gene. The results were expressed as fold induction.

To check primer specificity, representative PCR products were electrophoresed on a 1.5% agarose gel and visualized by illumination under UV light to confirm a single band at an
anticipated length. To confirm the correspondence between the nucleotide sequences of CYP 1B1, AhR1, and HO-1, cDNAs were amplified using Real-time-PCR (without melting step). Real-time PCR products were electrophoresed on a 1.5% agarose gel. DNA extraction from agarose gel was performed using Gel/PCR extraction kit (Nippon Genetics Europe GmbH, Dueren, Germany). The nucleotide sequences of the PCR products were determined using dye terminator removal kit (NIPPON GENETICS EUROPE GmbH). Then, they were subjected to an automated sequence analysis using ABI sequencer (model ABI Prism 3130, Applied Biosystems, TUAT, Tokyo, Japan) for the nucleotide sequences of CYP 1B1, AhR1, and HO-1. Nucleotide sequence comparisons were performed using the BLAST network program (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA).

Moreover, the efficiency of PCR among all target genes (>90%) was confirmed using serial dilutions of liver cDNA sample. Specific amplification of the target sequence in each real-time PCR reaction was further verified by melting curve analysis.

2.8. Statistical analysis

Data were tested for homogeneity distribution of variance using GraphPad Prism Version 5. The data were statistically analyzed using one-way analysis of variance followed by Tukey’s test at each time point in comparison to the control group at the same time. The statistical analyses were performed using the software program GraphPad Prism Version 5. All data were expressed as means ± S.E.M. A probability value of $P < 0.05$ was considered as significant.

3. Results

3.1. Effect of PNP on plasma corticosterone hormone

There was a significant increase in plasma concentrations of corticosterone at 45D, 60D, and 75D post PNP administration in treated birds when compared with the control values as shown in Fig. 1.

3.2. Effect of PNP on liver histology

Liver morphology and architecture were normal in the control group (Fig. 2A). On the other hand, in the low dose group, the PNP treatment induced focal lymphocytes infiltrations (Fig. 2B). The mid dose group showed degeneration of hepatocytes with some necrotic cells, and perilobular cirrhosis (Fig. 2C). In the high dose group, multi areas of congestion of blood vessels and focal lymphocytic infiltration with mild hepatocytes degeneration were observed (Fig. 2D).

3.3. Effect of PNP on relative liver genes expression of selected enzymes in vivo at different times

The effect of $p$-nitrophenol on the expression of marker genes related to metabolism and oxidative stress in the liver (CYP 1B1, 1A4, 1A5 enzymes, AhR1, and HO-1) at 60D and 75D were shown in Fig. 3. The mRNA of these genes at 45D showed no significant changes (data not shown). At 60D, the PNP treated bird exhibited
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overexpression in CYP1A4 (~1.5-2-fold higher at high dose), 1B1 enzyme (~5-10-fold), AhR1 (~2-4-fold), and HO-1 (~2-fold) than CYP1A5, which showed no change when compared with value of the control group. With continuous PNP treatment the mRNA level of CYP1A4, 1A5, 1B1

Fig. 1. Plasma concentrations of corticosterone in Japanese quail treated with PNP at doses of 0, 0.01, 0.1, or 1 mg/kg/day for days 45 (45D), days 60 (60D) and days 75 (75D). Each bar represents the mean ± S.E.M of 5 quails per group. * P < 0.05, **P < 0.01 compared with control birds.

Fig. 2. Histopathology of liver sections of the control and the PNP-treated Japanese quails 75 days (75D) post PNP treatment. In the control group (A), the liver cell showed normal cell associations without any structural changes. In the 0.01 mg/kg (B), 0.1 mg/kg (C) and 1 mg/kg (D) PNP treated groups, the liver showed lymphocytic infiltration (black arrow), congestion of blood vessels (blue arrow) and degeneration of hepatocytes (green arrow). Scale bars = 100 μm.
enzymes, and AhR1 showed non-significant downregulation at 75D when compared with the values of the control group.

3.4. Effect of PNP on relative genes expression of selected enzymes and cell viability in vitro

In vitro direct effect of PNP on the CYP 450 enzymes system and oxidative stress enzyme in cultured hepatocytes were shown in Fig. 4. The mRNA expression encoding CYP1A5 was low at all groups of PNP treatments. Meanwhile, there were significant overexpressions in CYP1A4, CYP1B1, and HO-1 when compared with the control value. However, AhR1 mRNA was below the detection limit in both cell cultures. Regarding the effect of PNP treatments on cell viability, no significant adverse effect on cell viability in vitro at different PNP doses after 24 hr incubation was reported, except at 10 and 100 μM as the viability decreased significantly.
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when compared with the control positive values (untreated cells) as shown in Fig. 5.

4. Discussion

Organophosphorus (OPs) compounds are the most widely used group of pesticides all over the world. Recently, p-nitrophenol has incriminated in health problems for instance, dysfunction of both male and female reproductive system and vasodilatation of blood circulation. To our best knowledge, this is the first study to investigate the effect of chronic PNP as one of the major metabolite of some OPs insecticides on the liver metabolizing enzymes. To clarify the mechanism of PNP induced liver toxicity, groups of Japanese quails were treated with different doses of PNP.

Plasma corticosterone showed increase at 45D, 60D, and 75D post treatment in the PNP treated groups. These results are in agreement with a previous study by Li et al. (2009), which reported that 14 days of PNP treatment induced an increase of plasma corticosterone in immature rats. Previous studies have reported that PNP has estrogenic activity in vivo and in vitro. Furthermore, estradiol treatment in drinking water increases plasma corticosterone concentrations in male rats, so the increase in the corticosterone level may be attributed to the estrogenic activity of PNP or the stress condition due to PNP toxicity.

Liver CYP 450 includes a multigenic
superfamily, which encodes numerous enzymes. These liver enzymes play an important role in metabolizing a wide range of endogenous and exogenous compounds through the AhR to facilitate their solubilization and elimination. Hence, it is crucial to control their expressions. Some xenobiotics cause induction of isoforms involved in their metabolism. Conversely, other xenobiotics and several pathophysiological conditions such as inflammation suppress the expressions of many isoforms. Two major CYP1A subfamily enzymes, CYP1A4 and CYP1A5, were purified from birds’ livers. CYP1A4 and CYP1A5 mRNA expressions were used for quantification of CYP enzymes changes in birds. CYP1A4 exhibits catalytic specificity for 7-ethoxyresorufin deethylase (EROD) and aryl hydrocarbonhydroxylase (AHH), while CYP1A5 is mainly active in the epoxygenation of the endogenous fatty acid and arachidonic acid but it is inactive in EROD or AHH activities.

Moreover, Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor. AhR-regulated genes bind to AhR ligand to form a complex, which translocates to the nucleus and binds to AhR nuclear translocator (ARNT), then activates the xenobiotic response element. In turn, it modulates transcription of many AhR-regulated genes such as CYP 450. In vivo, after 60 days of PNP administration, birds exhibited overexpressions of CYP1A4, 1B1 enzyme, AhR1, and HO-1 with no significant changes in the CYP1A5. However, with continuous PNP administration, down-regulation of liver tested genes was reported. This observation is in agreement with a previous research, which reported that in vitro exposure of hepatic stellate cells to ethanol (EtOH), CYP1A and 1B1 mRNAs were increased due to induced AhR nuclear translocation and reduced AhR expression. Moreover, this research clarified that there was time-related decline in AhR expression. Moreover, This diminished CYP genes with continuous EtOH exposure. This clarified that might be PNP worked mainly through AhR1. These results might be attributed to the synergistic effects of drug toxicity and polycyclic aromatic hydrocarbons.

Moreover, Davarinos and Pollenz (1999) found that Benzo (a) pyrene (typical AhR ligand suppressors) reduced AhR mRNA expression. This down-regulation was considered as an important mechanism regulated by AhR to overcome the toxicity. Indeed, the results revealed by Davarinos...
and Pollenz (1999)\(^{15}\) were in agreement with the present study, which demonstrated that long-term (75 days) exposure to \(p\)-nitrophenol diminished the capability of liver cell to express AhR1.

AhR is a transcription element involved in inflammation, cell proliferation, and death\(^{58}\). Schmidt et al. (1996)\(^{46}\) hypothesized that down regulation of AhR is a mechanism by which chronic exposure to AhR-ligand induced liver fibrosis and cirrhosis. They confirmed their hypothesis using livers of AhR-null mice. They detected areas of fibrosis and scattered foci of apoptosis. Also, one of the cellular responses, to oxidative stress in isolated hepatocytes, is reduction in the levels of mRNAs encoding the CYP \(450\)\(^{46}\). This is due to the decrease in transcriptional rates of these genes and reduction of hepatic CYP 1A expression during oxidative stress\(^{10}\). The fluctuation between increases and decreases in the CYP450 and AhR1 observed in the current study could be a part of an adaptive response made by the liver to minimize cell damage and overcome or adapt the toxic effect.

\textit{In vitro}, the mRNAs of both CYP1A4 and HO-1 were induced in a concentration-dependent manner by PNP in cultured hepatocytes even though AhR1 mRNAs was below the detection limit in both cell cultures. The discrepancy between the results might be attributed to the fact that mRNA of AhR in the culture was degraded rapidly because of the short half-life span (8 hr)\(^{7}\). Rapid loss of AhR was reported in cultured cells exposed to TCDD although the up regulation of CYP 1A1 levels remained high until 72 hr post-treatment, despite the very low nuclear AhR levels that existed at that time\(^{16}\). This might be attributed to a small fraction of nuclear AhR, which could be sufficient to drive genes related transcription\(^{16}\). Moreover, Davarinos and Pollenz (1999)\(^{20}\) found that AhR protein was rapidly depleted \textit{in vitro} following exposure to ligands.

In the present study, an overexpression of CYP1B1 was observed. CYP1B1 is responsible for the hydroxylation of estradiol-17\(\beta\) (E2)\(^{14,50}\). A previous article revealed that PNP exhibited estrogenic activity\(^{20}\). Therefore, the increase in CYP1B1 mRNA might activate the estrogen metabolism\(^{50}\).

Heme oxygenase (HO-1) is rate-limiting enzyme in the physiological degradation of heme\(^{8,13}\). It gives protection against programmed cell death and acts as a cellular adaptive resistance pathway\(^{13}\). Its expression can be modulated by xenobiotics and cellular oxidative stress factors\(^{8}\) such as arsenite. PNP caused a significant increase in HO-1 at mid and high doses in the \textit{in vivo} experiment after 60 days. Nevertheless, it returned to a normal level at 75D. Moreover, \textit{in vitro} hepatocyte cell culture showed a dose dependent increase in HO-1 and CYP1A4. Previously, Anwar-Mohamed et al. (2012)\(^{8}\) reported that HO-1 partially restored the enzymatic activity of these CYP 450 that was initially decreased by arsenite in isolated rat hepatocytes. This observation is in agreement with a previous study, which reported that the organic extract of DEPs showed induction of HO-1 gene expression\(^{27}\). Another previous study demonstrated that Hg\(^{2+}\) alone or in the presence of TCDD was able to increase liver HO-1 mRNA levels as early as 6 hr\(^{7}\). However, this increase was abolished after 24 hr, which might be attributed to the short half-life span of HO-1 mRNA and protein\(^{42}\).

In conclusion, PNP has the ability to disrupt liver health condition by affecting various CYP 450 enzymes and exhibit liver oxidative stress related enzymes. Consequently, on the long run, liver tries to overcome PNP toxicity and stress condition. The results of this research considered as a point forward towards understanding liver toxicity with PNP pollutant. Hence, PNP should be considered as a substance clearly involved in causing deleterious effects on liver health condition.
**Conflict of interest statement**

The authors declare that they have no conflict of interest.

5. References


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