Downregulation of male-specific cytochrome P450 by profenofos

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Abstract
The health hazards of individual organophosphorus insecticides have been characterized by their acute toxicity, mainly by investigating their cholinesterase inhibition. However, the chronic effects of most of these toxicants on the drug-metabolizing enzymes have not been investigated. Profenofos (O-4-bromo-2-chlorophenyl O-ethyl S-propyl phosphorothioate) is an organophosphorus pesticide widely used in cotton cultivation. In the present study, we investigated the effect of profenofos on male-specific cytochrome P450 (CYP) enzymes in adult Wistar rats. We orally administered 17.8 mg/kg body weight, twice weekly for 65 days. Profenofos downregulated levels of hepatic and testicular CYP2C11 and CYP3A2 mRNA and protein expression. Testicular aromatase (CYP19A) mRNA was decreased in the profenofos-treated rats compared to controls. Overall, the present study suggests that profenofos acts as an endocrine disruptor of male-specific CYP enzymes and affects testosterone concentration, which implicates its deleterious effects on animal or human males chronically exposed to organophosphorus pesticide.

Key Words: aromatase, CYP3A2, CYP2C11, cytochrome P450, profenofos

Introduction
The wide use of organophosphorus (OP) pesticides in agriculture has several interesting implications for environmental safety, such as their persistence and selective toxicity for insects rather...
than mammals. However, in spite of the selectivity for insects, OP pesticides are often highly toxic to humans and are responsible for most accidental intoxications in agriculture and the pesticide industry. Besides occupational exposure to relatively high doses, the general human and animal population may be chronically exposed to OP pesticides, primarily due to their presence as residues in food and drinking water.

Profenofos (O-4-bromo-2-chlorophenyl O-ethyl S-propyl phosphorothioate) is a broad-spectrum OP miticide that is used heavily in cotton-growing areas. Batley and Peterson ranked its risk in the mid-range on a list of 12 prominent cotton pesticides. Profenofos is reported to be highly toxic to some aquatic organisms, but only limited ecotoxicological data are available. OP pesticides require sulphoxidation, which is catalyzed by cytochrome P450 (CYP). This reaction yields unstable phosphooxythiiran intermediates, which are degraded upon desulphuration to the corresponding phosphate triesters or oxons. These compounds exert their toxic effects by powerful inhibition of brain acetylcholinesterase (AChE). Profenofos is also oxidized by the monooxygenase system to form AChE inhibitors, and its bioactivation varied with the pesticide substrate and the CYP isozyme involved.

Evidence of a role for sulphoxidation in the differential toxicity of OP pesticides in mammals is based on in vitro data and is not clear. Parathion sulphoxidation in human liver has been traced mainly to the actions of CYP3A4 and CYP2B6, and wide variability in this activity has been found in human liver samples. In humans, CYP3A4 activity is predominant at high OP pesticide concentrations.

In previous study, we showed that the profenofos altered plasma testosterone concentration and testis function in male rats. Profenofos administration increased expressions of testosterone generating CYP enzymes. The previous results lead us to direct our attention to testosterone metabolizing CYP isoforms, since CYP-dependent hydroxylation of steroid hormone also contributes to the homeostasis of steroid hormone levels. The rat liver contains at least a dozen sex-dependant isoforms of CYP that are regulated by sex-dependant profiles of circulating growth hormone (GH), and male dominant or specific CYP isoforms contribute to the testosterone metabolism. CYP3A2 is expressed in the liver of both female and neonatal male rats, but the levels drop below detectable limits at puberty in females, while they are maintained throughout life in males. CYP2C11 accounts for approximately one third of all CYPs in male rat liver, while it is essentially undetected in female rat liver. The male-specific CYP2C11 metabolizes a host of xenobiotics such as benzphetamine, aminopyrine, ethylmorphine, benzo[a]pyrene and warfarin. The downregulation of CYP2C11 by various agents is primarily due to a decrease in its mRNA expression, which is followed by a similar decrease in protein levels.

Aromatase is a product of a unique gene called CYP19. Aromatase is the terminal enzyme involved in the irreversible transformation of androgens into estrogens, and this microsomal enzyme complex comprises a specific heme-glycoprotein (P450-arom) that functions using ubiquitous reductase as an electron donor. P450 aromatase plays a role in development, reproduction, sexual differentiation and behavior, as well as in homeostasis of bone, lipid metabolism, brain functions, and diseases such as breast and testicular tumors. It is difficult to find a tissue completely devoid of aromatase gene expression. In the mammalian testis, aromatase is mainly localized in the Leydig cells. It was reported that aromatase inhibitors reduce spermatid maturation in rats and monkeys, and the disruption of spermatogenesis appears specifically to affect early round spermatid differentiation in adult male mice deficient in aromatase. Further, it has been reported that sub-lethal doses of pesticides led to alterations in reproductive performance in birds and mammals.
not fully elucidated. In this study, we investigated the effects of chronic exposure to profenofos on male fertility by determination of its effect on the male-specific CYPs in the liver and testis of male laboratory rats.

Materials and Methods

Materials: Profonfos was purchased from Syngenta, Ciba-Geigy (Geiza, Egypt). Goat anti-rat CYP2C11 and rabbit anti-rat CYP3A2 antibodies were from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA), rabbit horseradish peroxidase-labeled anti-goat IgG and diaminobenzidine tetrahydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Goat horseradish peroxidase-labeled anti-rabbit IgG was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Animals and treatments: Wistar rats (16-week-old, SLC, Hamamatsu, Japan) were housed in translucent cages at 24 ± 1°C with 12 hr light and 12 hr dark cycle, and given laboratory feed and water ad libitum. They were divided into 2 groups: 3 controls and 3 treated with profenofos. Treatment of all animals was performed according to the Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Veterinary Medicine, Hokkaido University. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University. Treated rats were given 17.8 mg/kg profenofos orally twice weekly for 65 days²⁵ and the control rats were given the vehicle, distilled water. Twenty-four hours after the last dose, the rats were sacrificed with carbon dioxide, and livers and testes were removed. The microsomal fractions from livers and testis were prepared according to the method of Omura and Sato²⁷ with slight modifications. Liver and testis were minced and homogenized in 3 volumes of ice-cold 1.15% potassium chloride solution with a Teflon homogenizer. Homogenized samples were centrifuged at 9,000 g at 4°C for 20 min. The supernatant fraction was centrifuged at 105,000 g at 4°C for 70 min to obtain a mitochondria-free microsomal pellet. The washed microsomes were then suspended in 0.1 M potassium phosphate buffer, pH 7.4, divided into 1.5 ml tubes, frozen in liquid nitrogen and kept at -80°C until use. Microsomal protein concentrations were determined according the method of Lowry et al.¹⁸ using BSA as a standard.

Western blot analysis: For quantification of CYP2C11 and CYP3A2, 12 µg aliquots of liver microsomal protein from treated and control rats were applied to 10-12% sodium dodecyl sulfate (SDS) polyacrylamide gels and separated by electrophoresis using a Protein 2 mini 1-D cell (Bio-Rad, Hercules, CA, USA)³⁵. Western blot analysis was performed according to the method of Towbin et al.³⁴. The proteins were electrotheretically transferred to nitrocellulose membranes, blocked in 5% skim milk in phosphate suffer saline (PBS) containing 1% Tween 20 for 2 hr at room temperature and probed with polyclonal goat anti-rat CYP2C11 or polyclonal rabbit anti-rat CYP3A2 antibody in PBS containing 1% Tween 20 on a shaker for 2 hr at room temperature. Horseradish peroxidase-labeled anti-goat IgG or anti-rabbit IgG, respectively, was used as the secondary antibody. Immunoreactive protein bands were visualized colorimetrically by oxidation of 0.025% 3,3 diaminobenzidine tetrahydrochloride with 0.0075% hydrogen peroxide catalyzed by peroxidase in 50 mM Tris-HCl (pH 7.6). Intensities of immunoreactive bands were densitometrically analyzed on a Macintosh computer using the public domain U.S. National Institutes of Health Image program available on the internet at (http://rbs.info.nih.gov/nih-image/).

RNA extraction: Total RNA was isolated from 50 mg of liver and testis using TriReagent (Sigma-Aldrich). Briefly, liver and testis tissues were homogenized in 1 ml TriReagent, and then 0.3 ml of chloroform was added to the sample. The mixture
was then shaken for 30 sec, followed by centrifugation at 15,000 g at 4°C for 20 min. The supernatant layer was transferred to a tube, and an equal volume of isopropanol was added; the samples were shaken for 15 sec and centrifuged at 4°C and 15,000 g for 15 min. The RNA pellet was washed with 70% ethanol and dissolved in 0.1% diethylpyrocarbonate (DEPC) in water. The prepared RNA was electrophoresed to demonstrate the RNA integrity, and then the optical density was measured on a spectrophotometer. The optical density of all RNA samples was 1.7 to 1.9 based on the 260/280 ratio.

**RT-PCR**: A mixture of 5 µg of total RNA and 0.5 ng oligo dT primer in a total volume of 24 µl sterile ultrapure water was incubated at 70°C for 10 min, then removed from the thermal cycler (iCycler, BioRad). A mixture of 8 µl (5X) RT-buffer, 2 µl 10 mM dNTP, 2 µl DEPC water and 2 µl reverse transcriptase (Toyobo Co., Ltd., Osaka, Japan) was added and it was incubated in a thermal cycler at 30°C for 10 min, 42°C for 1 hr and 90°C for 10 min. For semi-quantitative PCR, 1 µl aliquots of the synthesized cDNA were added to 20 µl of a mixture containing sterilized ultra-pure water, 2 µl of 10X PCR buffer, 2 µl of dNTP (2.5 mM), 0.3 µl of sense and anti-sense primers (10 µM) and 0.1 µl of Taq polymerase (Takara, Kyoto, Japan). Amplification of each gene was initiated by 1 cycle of denaturation at 95°C for 1 min, followed by cycles of denaturation at 94°C for 1 min, annealing at the proper temperature for 1 min and extension at 72°C for 1 min for the proper number of cycles using a DNA thermal cycler (iCycler). Cycle numbers were determined to be in the linear range for CYP2C11, CYP3A2, aromatase and G3PDH (see Figure legends). The primer sequences used are shown in Table 1. The samples were finally incubated for 7 min at 72°C after the last cycle of amplification. The amplified PCR products were separated by electrophoresis on a 1-1.5% gradient of agarose gel. Bands of DNA were stained with ethidium bromide and visualized by ultraviolet illumination. Photographic images were converted to computer files with an Epson color-image scanner in combination with Adobe Photoshop 6.0 software. The amounts of CYP mRNA were normalized to the corresponding bands of G3PDH and expressed relative to the control level.

**Statistical analysis**: All data are expressed as means±SD. An unpaired t-test was used to compare the two means of the two groups. P values less than 0.05 were considered statistically significant.

**Results**

**Effects of profenofos on CYP3A2 protein and mRNA in rat liver and testis**

Treatment of adult male Wistar rats with 17.8 mg/kg profenofos, which is 10% of the LD50, for 65 days decreased the hepatic CYP3A2 protein about 30% and downregulated hepatic CYP3A2 mRNA

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Table 1. Primers used in amplification of genes by semi-quantitative PCR
by 60% (Fig. 1). To further investigate the sites of profenofos effects, measurement of CYP3A2 in the testis showed that testicular CYP3A2 was decreased to less than 40% of CYP3A2 protein and mRNA in the controls following the pattern of the changes in hepatic CYP3A2 (Fig. 2).

Effects of profenofos on CYP2C11 protein and mRNA in rat liver and testis

In adult rats, CYP2C11 is expressed only by males. Profenofos treatment produced a more than 50% decrease in the hepatic CYP2C11 protein and mRNA (Fig. 3), which indicates the ability of profenofos to down-regulate CYP2C11 expression.

Fig. 1. Downregulation by profenofos of CYP3A2 expression in rat liver. Male Wistar rats were given 17.8 mg/kg body weight profenofos orally twice weekly for 65 days. A. Hepatic microsomal protein samples (12 µg/lane) were applied to 10% SDS-PAGE, transblotted onto nitrocellulose membranes and reacted with CYP3A2 antibodies as described in Materials and Methods. Then, the intensities of the CYP3A2 protein signals were calculated in relation to controls. B. DNA bands of CYP3A2 and G3PDH mRNA. Total RNA was isolated, and RT-PCR was performed as described in Materials and Methods. cDNA samples were amplified in duplicate for 30 cycles for CYP3A2, lower panel, and 25 cycles for G3PDH mRNA, upper panel. Then, CYP3A2 mRNA was normalized to the corresponding bands of G3PDH and analyzed relative to the control level. Each treatment is represented by 3 rats. Data are represented as mean ± SD. *Lower than control level, P < 0.05.

Fig. 2. Downregulation by profenofos of CYP3A2 expression in rat testis. Male Wistar rats were given 17.8 mg/kg body weight profenofos orally twice weekly for 65 days. A. Testicular microsomal proteins (20 µg/lane) were applied to 10% SDS-PAGE, transblotted onto nitrocellulose membranes and reacted with CYP3A2 antibodies as described in Materials and Methods. One testis of the 3rd rat of the control group was pooled with both testes of the other two rats. CYP3A2 protein signals were calculated relative to controls. B. DNA bands of CYP3A2 and G3PDH mRNA. Total RNA was isolated, and RT-PCR was performed as described in Materials and Methods. cDNA samples were amplified in duplicate for 32 cycles for CYP3A2, lower panel, and 25 cycles for G3PDH mRNA, upper panel. CYP3A2 mRNA was normalized to the corresponding bands of G3PDH and analyzed relative to the control. Each treatment is represented by 3 rats. Data are represented as mean ± SD. *Lower than control level, P < 0.05.
fenofos to disrupt the transcription of hepatic CYP2C11. We further measured the testicular CYP2C11, which also followed the trend in the liver. Profenofos treatment downregulated the expression of CYP2C11 protein about 60%, and CYP2C11 mRNA was about 20% of the control level (Fig. 4).

**Effect of profenofos on testicular aromatase mRNA in rat**

We also measured the expression level of testicular aromatase. The results demonstrate that profenofos treatment produced a greater than 80% decrease in the testicular aromatase mRNA expression compared to controls (Fig. 5).

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**Fig. 3. Downregulation by profenofos of CYP2C11 expression in rat liver.** Male Wistar rats given 17.8 mg/kg body weight profenofos orally twice weekly for 65 days. A. Hepatic microsomal protein samples (12 µg/lane) were applied to 10% SDS-PAGE, transblotted onto nitrocellulose membranes and reacted with CYP2C11 antibodies as described in Materials and Methods. Then, CYP2C11 protein signals were calculated relative to the control. B. DNA bands of CYP2C11 and G3PDH mRNA. Total RNA was isolated and RT-PCR was performed as described in Materials and Methods. cDNA samples were amplified in one PCR tube for 24 cycles. Then, CYP2C11 mRNA, lower panel, was normalized to the corresponding bands of G3PDH, upper panel, and analyzed relative to the control. Each treatment is represented by 3 rats. Data are presented as mean ± SD. *Lower than control level, P < 0.05.

**Fig. 4. Downregulation by profenofos of CYP2C11 expression in rat testis.** Male Wistar rats were given 17.8 mg/kg body weight profenofos orally twice weekly for 65 days. A. Testicular microsomal proteins (20 µg/lane) were applied to 10% SDS-PAGE, transblotted onto nitrocellulose membranes and reacted with CYP3A2 antibodies as described in Materials and Methods. One testis of the 3rd rat of the control group was pooled with both testes of the other two rats. CYP3A2 protein signals were calculated relative to the control. B. DNA bands of CYP2C11 and G3PDH mRNA. Total RNA was isolated, and RT-PCR was performed as described in Materials and Methods. cDNA samples were amplified in one PCR tube for 28 cycles. Then, CYP2C11 mRNA, upper panel, was normalized to the corresponding bands of G3PDH, lower panel, and analyzed relative to the control level. Each treatment is represented by 3 rats. Data are represented as mean ± SD. *Lower than control level, P < 0.05.
**Discussion**

Exposure to foreign compounds may cause changes in endocrine function both directly (as hormone agonists or antagonists) and indirectly (altering circulating levels of hormones by influencing rates of hormone synthesis or metabolism), which can severely affect steroid hormone actions. In previous study, we found that the increased concentration of plasma testosterone and enlarged Leydig cells in rat testis after the exposure to profenofos. We suggested the possibility that induction of CYP isoform expressions, which metabolize androgens, might be caused to accelerate the elimination of testosterone. Our study was designed to investigate the effects of profenofos, a phosphorothioate organophosphorus compound, on male fertility by exploring its effect on male-specific hepatic and testicular CYPs.

In our study, treatment of male Wistar rats with profenofos twice a week for 65 days downregulated the hepatic CYP3A2 protein, accompanied by a reduction in its mRNA. The downregulation of CYP3A2 may indicate its role in the metabolism of profenofos because it was reported that human CYP3A4 is predominantly active at high OP pesticide concentrations. The long (65 days) treatment of the rats in our experiment might lead to accumulation of profenofos in the body. During OP desulfuration, activated sulfur atoms are formed that bind irreversibly to the specific CYP isoforms that catalyze the reaction, resulting in a time-dependent reduction of the related enzymatic activity.

CYP3A is regulated mainly by the pregnane X receptor (PXR), which is now known to be the key factor in the oxidative metabolism of xenobiotics, through CYP3A and CYP2B induction, and also in their conjugative metabolism and transport. The downregulation of CYP3A in the liver could implicate profenofos by interfering with PXR function. However, the results of a previous report by Zhang et al. showed that PXR is not expressed in rat testis, while CYP3A2 was demonstrated to be expressed in rat testis. The ability of profenofos to downregulate CYP3A2 mRNA in the testis as well as the liver may indicate the ability of profenofos to affect CYP3A2 at the transcription level independently of PXR.

In our study, treatment of male Wistar rats with profenofos downregulated the hepatic CYP2C11 mRNA and protein levels compared to those of the control (Fig. 1). These results may indicate the involvement of CYP2C11 in profenofos metabolism, and they are consistent with a report that showed that CYP2C11 contributes to the metabolism of the organophosphorus pesticide diazinon. Hepatic CYP2C11 expression is regulated by the male pattern of GH secretion through the Janus-kinase 2 signal transducer and activators of the transcription protein (JAK/STAT5b) signal transduction pathway. In our study, the downregulation of CYP2C11 expression at the levels of

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**Fig. 5. Downregulation by profenofos of testicular aromatase expression in rats.** Male Wistar rats were given 17.8 mg/kg body weight profenofos orally twice weekly for 65 days. DNA bands of aromatase mRNA and G3PDH mRNA. Total RNA was isolated and RT-PCR was performed as described in Materials and Methods. cDNA samples were amplified in two PCR tubes for 36 cycles for aromatase, lower panel, and 25 cycles for G3PDH mRNA, upper panel. Aromatase mRNA bands were normalized to the corresponding bands of G3PDH and analyzed. Each treatment is represented by 3 rats. Data are represented as mean ± SD. *Lower than control level, P < 0.05.
mRNA and protein may indicate the ability of profenofos to disturb the GH-JAK/STAT5b-CYP2C11 pathway through its effect on the pattern of GH secretion. The plasma pattern of GH secretion directs the sex-specific expression of this enzyme: CYP2C11 is induced by a pulsatile pattern of GH in the plasma, and the expression of CYP2C11 enzyme is downregulated by a continuous level of GH in the plasma. The downregulation of CYP2C11 by the OP pesticide in our study may disturb the GH level or pattern of secretion, because it was reported that paraoxones disturb pituitary GH and prolactin secretion. The downregulation of CYP2C11 at the mRNA and protein levels in both liver and testis may implicate induction of a negative transcriptional mechanism of CYP2C11 by the phosphorothioate profenofos.

The treatment with profenofos also led to downregulation of testicular aromatase in rat (Fig. 5) in treated rats compared to the controls. The irreversible conversion of androgens into estrogens is catalyzed by aromatase, a product of a unique gene called CYP19. Our result indicated a possibility of reduced estradiol production. It is well known that normal testicular development and maintenance of spermatogenesis are controlled by gonadotropins and testosterone, whose effects are modulated by locally produced factors, and among them, estrogens are obviously involved. It has been shown that infertility in ArKO (aromatase knockout) mice is consequent to an impairment of spermiogenesis associated with a decrease in sperm motility and an inability to fertilize oocytes.

Phosphorothioate (parathion) was reported to be a potent inhibitor of the male-specific CYPs CYP2C11 and CYP3A2 in rat liver. In this study we present data showing that treatment of Wistar rats with profenofos (another phosphorothioate) downregulated male-specific hepatic and testicular cytochrome P450, mainly CYP3A2, CYP2C11 and testicular aromatase. We could suggest that the alterations of these CYP expression levels were finally due to regulate the elevated testosterone concentration. The overall results suggest that the phosphorothioate profenofos is an endocrine disruptor especially in males.

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