マウス肝ミクロソームのチトクロームP-450に対する有機リン殺虫剤

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Effect of Organophosphorus Insecticides on Hepatic Microsomal Cytochrome P-450 in Mice

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A single intraperitoneal injection of fenitrothion (100 mg/kg) to mice brought a biphasic effect on microsomal drug metabolism and cytochrome P-450 content. A slight decrease in protoheme was observed, whereas fenitrothion did not affect microsomal cytochrome b_5 content.

Pretreatment of mice with SKF 525-A, an inhibitor of drug metabolism, afforded some prevention against the decrease in cytochrome P-450 owing to fenitrothion injection. On the other hand, there was the marked decrease in cytochrome P-450 when some organothiophosphates were incubated with microsomes under the presence of NADPH-generating system *in vitro*. The decrease was observed as long as organophosphates contained P=S group.

These results reveal that the depression of cytochrome P-450 in an earlier stage might be coupled with the oxidative metabolism of sulfur compound. Recovery from the decrease in cytochrome P-450, which ordinary take place within 24 hr, was delayed by the concomitant treatment of mice with cobalt chloride indicating that the heme synthesis may be involved in recovery and rebounded increase in cytochrome P-450 in the later stage after the treatment with fenitrothion.

INTRODUCTION

Evidence has been reported recently that various organophosphorus insecticides inhibit drug-metabolizing enzymes in liver.[1-5] Stevens and Greene[6] have indicated that the inhibitory effect of organophosphates is associated with their ability to bind to cytochrome (Cyt.) P-450. In addition, Stevens and Greene[7] have shown that administration of parathion to mice resulted in the decrease of Cyt. P-450 content in the early time period and the delayed increase of Cyt. P-450 at 48 hr.

Authors also reported the inhibition of drug metabolism and the marked decrease in Cyt. P-450 content during the early period after the injection of fenitrothion.[8] And, furthermore, we suggested that this decrease might be correlated to the metabolism of fenitrothion, since the pretreatment of mice with phenobarbital resulted in further decrease in Cyt. P-450 by subsequent administration of fenitrothion.

On the other hand, we have shown[9] that the administration of fenitrothion to mice induces hepatic δ-aminolevulinic acid synthetase (ALA synthetase), the first enzyme in heme synthesis, suggesting that the increased availability of heme in the liver through the induction of ALA synthetase might be associated with the recovery of P-450 content from the lowered level.

In order to ascertain the view points mentioned above, the present studies dealt with the effect of pretreatment with SKF 525-A, an inhibitor of drug metabolism, on the action of fenitrothion and the effect of organophosphorus insecticides *in vitro* on Cyt. P-450 content.

In addition, the effect of an administration of cobalt chloride, which is known to inhibit heme synthesis[10] and to induce heme oxygenase,[11] in combination with fenitrothion on

Cyt. P-450 content was examined.

EXPERIMENTAL

1. *Materials*

Insecticides employed were fenitrothion, parathion and paraoxon which were kindly supplied from Sumitomo Chemical Co., Ltd., and diazinon which was a gift from Nippon Kayaku Co. Glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. SKF 525-A was purchased from Smith Kline and French Labs., Ltd. Other chemicals were of reagent grade.

2. *Animals and Treatment*

Male ddY mice, weighing 25-28 *g*, and male Wistar rats, weighing 200 *g*, were used. Fenitrothion was given intraperitoneally at a dose of 100 mg/kg. In some experiments, mice were treated intraperitoneally with SKF 525-A (40 mg/kg) or subcutaneously with cobalt chloride (100 mg/kg), dissolved in 0.9% NaCl, prior to or simultaneous with the administration of fenitrothion. Control mice were injected with the vehicle only.

3. *Preparation of Microsomes*

Mice were sacrificed and livers were rapidly excised, perfused with cold 1.15% KCl and homogenized in 5 vol of 1.15% KCl containing 0.1 mM EDTA. Microsomes were prepared from 9,000 *g* supernatant fraction by centrifugation at 105,000 *g* for 1 hr, followed by washing with 1.15% KCl-0.1 mM EDTA. The resulting microsomal pellets were resuspended in 0.1 M phosphate buffer (pH 7.0) to a protein concentration of 2 mg/ml.

4. *Analyses*

N-Demethylation of aminopyrine was determined by the method of Cochin and Axelrod.[12] Hydroxylation of aniline was measured by the method of Imai *et al.*[13] Contents of Cyt. P-450, Cyt. b_5 and protoheme in microsomes were determined according to the method of Omura and Sato.[14] Protein content was measured by the method of Lowry *et al.*[15] with bovine serum albumin as a standard.

RESULTS

1. *Effect of Fenitrothion on Microsomal Drug Metabolizing Enzyme Activity, Cyt. P-450, Cyt. b_5 and Protoheme Content*

Figure 1 shows the time dependent effect of fenitrothion on microsomal drug metabolizing enzyme activity and Cyt. P-450. At the early period, that is 2 to 4 hr after a single administration, Cyt. P-450 showed marked decrease, but returned nearly to control level within 24 hr. Unexpectedly, the increment of P-450 (approx. 1.3 times of control) was observed at 36 hr after dosing. This change in Cyt. P-450 content was well coincided with aminopyrine demethylation activity, and compatibile with the observation of Stevens and Greene[7] who reported the increase of P-450 at 48 hr after the acute exposure to parathion. These results indicate that fenitrothion produces a biphasic effect on hepatic drug metabolizing enzyme system in mice.

In contrast to Cyt. P-450, Cyt. b_5 content in

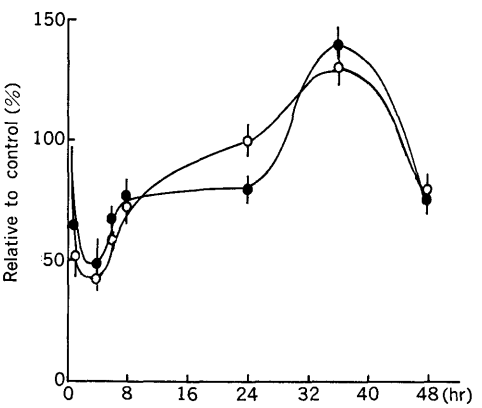

Fig. 1 Time course of the effect of fenitrothion on hepatic microsomal aminopyrine *N*-demethylation and cytochrome P-450 content in mice.

Mice were injected intraperitoneally with fenitrothion (100 mg/kg) and sacrificed at the times indicated. The average enzyme activity and cytochrome P-450 content of control group were 64.7±10.9 nmol HCHO/mg protein/10 min and 0.950±0.036 nmol/mg protein, respectively. The vertical bars are standard errors of the means of three to five experiments.

—○—: aminopyrine demethylation, —●—: cytochrome P-450.

Table 1 Effect of fenitrothion on hepatic microsomal cytochrome b_5 and protoheme content in mice.

Treatment	Cytochrome b_5 (nmol/mg protein)	Protoheme (nmol/mg protein)
Control	0.436±0.068	1.639±0.068
Fenitrothion	0.425±0.065	1.430±0.041

Mice were injected intraperitoneally with fenitrothion (100 mg/kg) 2 hr prior to sacrifice. Each value is the mean ±S.E. of four mice.

microsomes remained unchanged even at 2 hr after fenitrothion treatment. On the other hand, protoheme content in microsomes slightly decreased by the administration of fenitrothion indicating that the decrease in Cyt. P–450 content due to fenitrothion administration might be associated in some extent with the loss of protoheme (Table 1).

2. Effect of SKF 525-A on the Action of Fenitrothion

The previous result[8] has shown that the actual decrease in Cyt. P–450 content by fenitrothion was enhanced when phenobarbital was pretreated to mice. To confirm whether the decrease in Cyt. P–450 really relates to the metabolism of fenitrothion itself, SKF 525-A was employed as pretreating agent. Table 2 shows that under the defective drug metabolism the level of Cyt. P–450 remained about 80% of control at 2 hr after fenitrothion

Table 2 Effect of SKF 525-A on the action of fenitrothion.

Pretreatment	Treatment	Cytochrome P–450 (nmol/mg protein)	Aminopyrine demethylation	Aniline hydroxylation
Control	Control	0.960±0.056	9.60±1.13	7.16±0.37
SKF 525-A	Control	1.003±0.124	2.36±0.06	3.37±0.39
SKF 525-A	Fenitrothion	0.770±0.060	2.26±0.21	1.62±0.39
	SKF 525-A+ Fenitrothion	0.740±0.044	2.95±1.51	1.71±0.41

Mice were injected with SKF 525-A (40 mg/kg i.p.) 1 hr prior to or simultaneous with fenitrothion (100 mg/kg i.p.). Mice were killed 2 hr after the administration of fenitrothion. Drug-metabolizing activities were determined by 9,000 *g* supernatant fraction and expressed as the metabolites produced in nmol/mg protein/30 min. Each value is the mean ±S.E. of four to six mice.

Table 3 *In vitro* effect of organophosphorus insecticides on the concentration of hepatic microsomal cytochrome P–450 of mouse.

Insecticide added (M)		Cytochrome P–450 (nmol/mg protein)	
		+NADPH	−NADPH
None		0.976±0.058	1.011±0.005
Fenitrothion	1×10^{-4}	0.599±0.028	0.956±0.011
	1×10^{-5}	0.771±0.019	
None		0.910±0.019	
Diazinon	1×10^{-4}	0.724±0.023	
	1×10^{-5}	0.835±0.023	
Parathion	1×10^{-4}	0.588±0.051	0.841±0.038
	1×10^{-5}	0.778±0.004	
Paraoxon	1×10^{-4}	0.939±0.028	1.038±0.020
	1×10^{-5}	0.942±0.032	

Mouse liver microsomes (2 mg/ml) were incubated aerobically with insecticides in 30-ml conical flasks for 15 min. with and without NADPH generating system consisted of 5 μmol NADP, 30 μmol G–6–P and 5 units of G–6–P dehydrogenase in 0.1 M phosphate buffer containing 0.1 mM EDTA. At the end of incubation, the mixture was cooled and cytochrome P–450 content was determined directly in the incubation mixture. Values are the mean ±S.E. of three determinations.

administration which led ordinarily about 50% decrease (Fig. 1). As a results it was confirmed that the effect of fenitrothion on lowering Cyt. P-450 content may be closely coupled with the metabolism of fenitrothion itself.

3. *Effect of Organophosphorus Insecticides on Cytochrome P-450 in vitro*

To obtain more direct evidence concerning the decrease in Cyt. P-450, effect of organophosphorus insecticides *in vitro* with or without NADPH-generating system was examined in Table 3. The decrease in Cyt. P-450 was seen in all the organophosphorus insecticides containing P=S group, though the extent of decrease differed according to each compound, under the presence of NADPH. However, the decreasing effect was hardly seen under the absence of NADPH and in the compound with P=O group, such as paraoxon.

Table 4 shows the results of the same examination made with rat liver microsomes in the presence of NADPH-generating system, and the results were the same as that with the use of mouse liver microsomes.

These results *in vitro* reveal that thiophosphate insecticide itself must be metabolized by drug metabolizing enzyme system prior to express the effect on Cyt. P-450, which are well compatible with the results of experiments *in vivo*.

Table 4 *In vitro* effect of fenitrothin, parathion and paraoxon on the concentration of hepatic microsomal cytochrome P-450 of rat.

Insecticide added (M)		Cytochrome P-450 (nmol/mg protein)
None		0.912±0.019
Fenitrothion	1×10^{-4}	0.573±0.020
	1×10^{-5}	0.844±0.018
Parathion	1×10^{-4}	0.435±0.004
	1×10^{-5}	0.799±0.006
Paraoxon	1×10^{-4}	0.868±0.004
	1×10^{-5}	0.921±0.006

Rat liver microsomes were incubated with insecticides in the presence of NADPH generating system. Experimental details were described in Table 3. Values are the mean ±S.E. of the mean of two or three determinations.

4. *Effect of Cobalt Chloride on the Action of Fenitrothion*

As shown in Fig. 1, lowered Cyt. P-450 and drug metabolism, which appeared in the early period after the fenitrothion dosing, return to normal level within 24 hr and further increased thereafter. Early recovery may be due to easy metabolic conversion of fenitrothion, but in the rebound increase in the later process the induction of ALA synthetase might be involved. In order to ascertain this viewpoint, effect of cobalt chloride, the inhibitor of heme synthesis, was examined in

Table 5 Effect of $CoCl_2$ on the action of fenitrothion.

Treatment	Cytochrome P-450 (nmol/mg protein)	Protoheme (nmol/mg protein)
Control	0.875±0.003	1.389±0.013
Fenitrothion	0.770±0.038	1.550±0.070
$CoCl_2$	0.686±0.050	1.109±0.019
Fenitrothion +$CoCl_2$	0.479±0.074	0.857±0.075

Mice were injected (i.p.) with fenitrothion (100 mg/kg), cobalt chloride (100 mg/kg) or both simultaneously, and sacrificed 24 hr after the injection. Each value is the mean ±S.E. of 4 to 6 experiments.

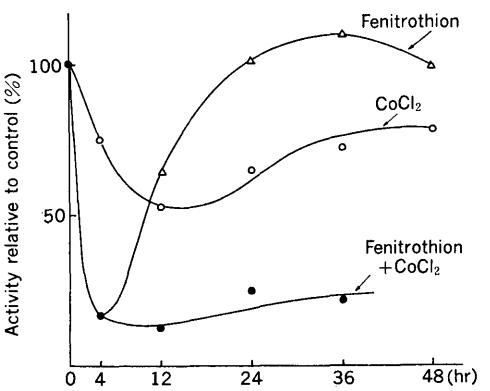

Fig. 2 Time course of aminopyrine demethylation activity in mouse liver after injection of fenitrothion and $CoCl_2$.

Treatment of animals was the same as Table 5. The control values of the activity were ranged from 26 to 30 nmol HCHO/mg protein/30 min.

Table 5. No recovery in both Cyt. P–450 and protoheme contents was observed at 24 hr after fenitrothion treatment by the concomitant administration of both cobalt chloride and fenitrothion. Aminopyrine demethylation activity was also remained suppressed even in the later period when the metabolic activity is to become rebounded maximum by the treatment of fenitrothion alone.

DISCUSSION

Numerous reports are available about the inhibition of drug metabolism by organophosphorus insecticides[1–5] and this inhibition was found to be due to the decrease in Cyt. P–450 content.[6] The effect seems to be preferential to Cyt. P–450 among microsomal components. Previous[8] and present results suggest that the metabolism of fenitrothion itself is necessary to decrease Cyt. P–450 *in vivo*. This contention could also be supported by the experiments *in vitro*. Namely, the presence of NADPH–generating system was essential for the decrease of P–450 when incubated with fenitrothion. All compounds having P=S group also resulted in the decrease of Cyt. P–450 in the presence of NADPH, whereas the effect was not observed by the compound with P=O group. Thus, the inhibition of drug metabolism by thiophosphates occurs while such compounds are metabolized themselves.

Norman *et al.*[16] concluded from their studies on parathion *in vitro* that the liberation and the binding of sulfur to microsomes during oxidative desulfuration might have an important significance. Furthermore, it has been reported that the administration of carbon disulfide to rats resulted in the marked and prolonged decline in drug metabolizing enzyme activities and Cyt. P–450 content.[17,18] Treatment of mice with carbon disulfide surely produced the similar results (data not shown). Recently, Dalvi *et al.*[19] have clearly shown that when carbon disulfide was metabolized *in vitro*, sulfur atom was released and bound to microsomes just as that seen by parathion. Thus, the binding of sulfur atom liberated from phosphorothioate insecticides to microsomes seems to be most likely to evoke the decrease in Cyt. P–450. However, further detailed examination will be necessary to prove how the sulfur can decrease Cyt. P–450.

Several investigators[20–22] have shown that the rapid decrease in Cyt. P–450 after the administration of drugs was due to the destruction of preexisting cytochrome rather than the inhibition of protein synthesis. Stevens[23] observed the loss of Cyt. P–450 and of total heme when some organophosphorus insecticides were incubated with microsomes in the presence of NADPH, suggesting the destruction of preexisting cytochrome.

The administration of fenitrothion also resulted in a slight decrease in the levels of protoheme in microsomes (Table 1), but fenitrothion did not produce any decrease in protoheme when preincubated with microsomes in the presence of NADPH-generating system, although a significant decrease of Cyt. P–450 was observed under these experimental conditions (data not shown).

Therefore, it is very likely that the binding of sulfur to microsomes may result in a preclusion of the binding of carbon monoxide to a portion of cytochromes, thus leading to an apparent decrease in the characteristic absorption spectra.

On the other hand, the present results seem to support the idea that the recovery and further increase in Cyt. P–450 produced by fenitrothion is associated with the increased availability of heme in the liver through a increase of ALA synthetase activity, since the administration of cobalt chloride, which would be expected to decrease the levels of heme in the liver by inhibiting heme synthesis or by inducing heme oxygenase, suppressed such a rebounding increase.

要　　約

フェニトロチオン 100 mg/kg をマウス腹腔に注射すると肝ミクロソームのチトクローム P–450 量の薬物代謝活性は 2 相性の変化を示す．プロトヘム量はやや減少するがチトクローム b_5 量は影響をうけない．

薬物代謝阻害剤の SKF–525A で前処理したマウスでは，フェニトロチオン投与による P–450 の減少度が少なくなる．一方，P=S結合をもつ有機リン剤をミクロソームと *in vitro* でインキュベートすると，NADPH の存在下に限りミクロソームの P–450 が著明に減少する．

このことは，有機リン剤投与で初期に起る P-450 の低下は S 化合物の酸化的代謝と共役していることを示す．有機リン剤投与後 24 時間くらいで P-450 量は平常に復するが $CoCl_2$ の前処理によりこの回復が遅延する．このことは，P-450 の回復およびそれにつづく増加現象にはヘム合成が関与していることを示すものである．

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