Potentialiation of EPN-Induced Inhibition of Liver Microsomal Carboxylesterase by Addition of Liver Cytosol from 6-Aminonicotinamide-Treated, Starved Rats

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Abstract—Addition of liver cytosol from 16 hr-starved rats treated with 6-aminonicotinamide to rat liver microsomes caused potentiation of the anti-carboxylesterase action of ethyl-p-nitrophenyl phenylphosphonothioate (EPN). This was not found when liver cytosol from non-pretreated rats after 16 hr-starvation was used. This potentiation of EPN-induced inhibition of carboxylesterase may be, at least in part, explained by the fact that treatment of rats with 6-aminonicotinamide resulted in a significant increase in NADPH level in liver cytosol which, in turn, stimulated formation of an EPN oxygen analog, a potent inhibitor of carboxylesterase, through cytochrome P-450-coupled monooxygenase.

Nonspecific carboxylesterase (EC. 3.1.1.1) in liver microsomes is well documented to be more susceptible to organophosphorus insecticides than acetylcholinesterase *in vivo* (1–6). We have already reported that addition of NAD to the incubation mixture containing both liver microsomes and EPN caused remarkable potentiation of carboxylesterase inhibition by EPN (7). In this study, it is important that pyridine nucleotide contents in liver cytosol are closely related to the liver microsomal drug metabolism which is coupled with cytochrome P-450.

On the other hand, 6-aminonicotinamide (6-AN) has been known to alter the content of pyridine nucleotides in liver, which may affect the pyridine nucleotide-dependent enzyme activities in liver tissue (8–10). Our previous paper (11) demonstrated that cytochrome P-450 content and aminopyrine N-demethylase activity in liver microsomes were increased by treatment of rats with 6-AN.

The present study was undertaken to investigate the changes in pyridine nucleotide contents in the liver cytosol of 6-AN-treated rats and the effect of the cytosol from 6-AN-treated rats on liver microsomal carboxyl-

esterase activity *in vitro*.

Materials and Methods

Animals: Adult male rats of the Wistar strain, weighing 200–250 g, were used. They were maintained on standard laboratory chow (Oriental Yeast Co., MF) and tap water *ad libitum* under a condition of constant room temperature (23–25°C) for at least one week before use.

Chemicals: EPN (analytical standard, 99+%) was purchased from Wako Pure Chemicals, Inc, Tokyo, Japan. Prior to use in the experiments, EPN was subjected to thin layer chromatography to remove the contaminants such as S-alkyl isomer and oxygen analog of EPN (EPN=O), and it was purified by recrystallization from chloroform:methanol (1:1, v/v). No contamination of the recrystallized product could be detected by thin layer chromatography on a silica gel plate (Kieselgel 60 F254) using iodide as the detection reagent. The solvent system used to develop these thin layer plates was n-hexane:chloroform:methanol (7:3:1, v/v). In this solvent system, EPN and EPN=O have R_f values of 0.69 and 0.44, respectively. An

authentic sample of EPN=O was prepared according to the method of Neal and DuBois (12) with minor modification by Satoh et al. (7).

Glucose 6-phosphate (G-6-P), G-6-P dehydrogenase (type VI), NADP, NADH and NADPH were purchased from Oriental Yeast Co., Tokyo; 6-aminonicotinamide and β -NAD⁺ (yeast, grade III) were from Sigma Chemical Co., St. Louis, MO; Isocarboxazid was kindly donated by Nippon Roche Co., Kamakura, Japan. All other chemicals were analytical or reagent grade.

Preparation of liver microsomes and cytosol: The rats were sacrificed by decapitation, their livers were perfused with 1.15% KCl to remove the blood and then homogenized in two volumes of 1.15% KCl. The 33% homogenate was centrifuged at $9,000 \times g$ for 20 min, and the supernatant obtained was recentrifuged at $105,000 \times g$ for 60 min to sediment microsomes. The postmicrosomal supernatant was used as a cytosol fraction in this study. The microsomes were washed once with 1.15% KCl and were used either the same day, or they were suspended in 1.15% KCl and stored at -70°C under nitrogen gas until used.

Enzyme assay: Carboxylesterase activity was determined using the colorimetric assay procedure based on conversion of isocarboxazid to benzylhydrazine as described previously (6). Activities of N-demethylase towards aminopyrine and ethylmorphine and activity of aniline hydroxylase were deter-

mined in terms of the formaldehyde formation by the method of Nash (13) and in terms of the formation of p-aminophenol by the method of Imai et al. (14), respectively.

Determination of pyridine nucleotide content: NAD and NADP contents were determined spectrophotometrically by measuring the increase in absorbance at 340 nm in the presence of either alcohol dehydrogenase or G-6-P and G-6-P dehydrogenase, respectively (15). NADH and NADPH contents were determined according to the procedure described above after conversion to the respective oxidized form.

Determination of EPN=O: EPN=O formed in the incubation mixture after 10 min-incubation was determined by the GC/MS method described in the previous paper (7).

Protein concentration was determined by the method of Lowry et al. (16).

All data in this study were statistically analyzed by Student's *t*-test.

Results

Enhancement of EPN-induced inhibition of microsomal carboxylesterase by liver cytosol in vitro: Inhibition of liver microsomal carboxylesterase by EPN (1×10^{-5} M) was clearly potentiated by addition of liver cytosol from non-treated, fed rats (Table 1). In contrast, there was almost negligible enhancement of EPN-induced inhibition of carboxylesterase when cytosol from rats starved for 16 hr (17:00–9:00) prior to sacrifice was added to the liver microsomes

Table 1. Effects of rat liver cytosol on EPN-induced inhibition of liver microsomal carboxylesterase

EPN	Cytosol added	Carboxylesterase activity
—	—	63.7 $\pm$ 3.5
+	—	56.6 $\pm$ 3.5
—	non-treated, fed	66.9 $\pm$ 11.8
+	non-treated, fed	5.8 $\pm$ 0.5*
+	non-treated, starved ¹⁾	62.0 $\pm$ 3.5
+	6-AN-treated, starved ²⁾	5.6 $\pm$ 0.4*

Carboxylesterase activity is shown as nmoles benzylhydrazine formed/mg protein/30 min. 1) Rats were starved for 16 hr prior to sacrifice, and cytosol was prepared. 2) 6-Aminonicotinamide (6-AN) was administered intraperitoneally to rats at a dose of 15 mg/kg 16 hr before sacrifice. The incubation mixture consisting of 2–3 mg protein of the microsomal pellet, 1×10^{-5} M of EPN, 0.5 ml of cytosol (protein approx. 10 mg) and 1 mM of isocarboxazid as substrate in 0.2 M Tris-HCl buffer (pH 8.0) was incubated at 37°C for 30 min following 10 min-preincubation in the absence of isocarboxazid. Each value is the mean $\pm$ S.E. from 7–8 experiments. * $P < 0.01$ vs. the EPN alone group.

from fed rat liver. These findings strongly suggested that diet-controlled component(s) in the cytosol may be involved in the potentiation, and pyridine nucleotides were considered as the compounds responsible for the potentiation.

On the other hand, 6-AN was injected intraperitoneally at a dose of 15 mg/kg, 16 hr prior to sacrifice, and its effect on the EPN-induced inhibition of carboxylesterase was examined.

Table 1 also showed that addition of liver cytosol from starved rats treated with 6-AN caused remarkable potentiation of carboxylesterase inhibition by EPN *in vitro*, while no significant change in carboxylesterase inhibition by EPN was observed when 6-AN itself was added in the incubation mixture containing EPN and liver microsomes (data not shown).

Pyridine nucleotide contents in liver cytosol: Rats were treated with 6-AN (15 mg/kg, i.p.) and subjected to 16 hr-starvation. They were sacrificed 16 hr later,

and the liver cytosol was prepared as described in the Materials and Methods. As shown in Table 2, NADPH contents in liver cytosol was remarkably decreased by starvation (group 2.), while 6-AN treatment caused a 2-fold increase in NADPH content and decrease in NAD content.

Changes in liver microsomal drug metabolizing enzyme activities by addition of liver cytosol: As shown in Table 3, a significant increase in aminopyrine N-demethylase activity was observed when the liver cytosol from non-treated, fed rats was added to the incubation mixture containing liver microsomes without added NADPH-generating system.

Determination of EPN=O content formed by addition of liver cytosol from 6-AN-treated, starved rats: In order to clarify the potentiation of EPN-induced inhibition of carboxylesterase by the cytosol, the amount of EPN=O formed was determined when liver cytosol from 6-AN-treated, starved rats were added to the incubation mixture con-

Table 2. Effects of 6-aminonicotinamide on pyridine nucleotide contents in rat liver cytosol

Pre-treatment	Pyridine nucleotide contents (nmoles/ml of cytosol)			
	NAD	NADP	NADH	NADPH
None (fed)	8.4±1.2	7.1±1.1	1.8±1.3	22.4±2.7
None (starved)	7.0±0.9	4.8±0.5	0.2±0.2	4.2±0.6**
6-AN (starved)	5.4±0.6*	5.7±1.1	0.5±0.4	9.0±1.6**#

Rats were starved and concurrently administered 6-AN intraperitoneally at a dose of 15 mg/kg 16 hr before sacrifice. Each value is the mean±S.E. from 7–10 animals. *P<0.05 and **P<0.01 vs. the non-treated, fed group. #P<0.05 vs. the non-treated, starved group. For details, see the legend to Table 1.

Table 3. Drug metabolizing enzyme activities in the presence or absence of EPN, cytosol and NADPH-generating system

Addition	Aminopyrine N-demethylase ¹⁾	Ethylmorphine N-demethylase ¹⁾	Aniline hydroxylase ²⁾
None	0.5±0.3	0.6±0.3	0.0±0.0
EPN alone	0.4±0.2	0.4±0.3	0.0±0.0
EPN+Cytosol	1.9±0.3*	1.1±1.0	0.1±0.1
Complete system ³⁾	58.9±6.4**	60.5±8.3**	16.6±2.0**

Concentration of EPN added was 1×10^{-5} M. Protein content of cytosol added was 3–5 mg per incubation mixture. 1) nmoles formaldehyde formed/mg protein/30 min. 2) nmoles p-aminophenol formed/mg protein/30 min. 3) Complete system consists of components usually used for assay of drug metabolizing enzyme activities, including the NADPH-generating system. Each value is the mean±S.E. from 3–5 experiments. *P<0.05 and **P<0.01 vs. the EPN alone group.

Table 4. Effects of liver cytosol on the enzymatic formation of EPN=O from EPN added to fed or starved microsomes of rat liver in vitro

Ms	EPN	Cytosol added	EPN=O formed ($\times 10^{-6}$ M)	% (EPN=O EPN added)
Fed	+	—	N.D.	<0.1
	+	non-treated, fed	2.80 $\pm$ 0.55	18.7
	+	non-treated, starved ¹⁾	0.64 $\pm$ 0.40*	4.3
	+	6-AN-treated, starved ²⁾	1.99 $\pm$ 0.88	13.3
	+	6-AN-treated, starved+SKF 525-A	N.D.	<0.1
Starved	+	—	N.D.	<0.1
	+	non-treated, fed	1.99 $\pm$ 0.26	13.3
	+	non-treated, starved ¹⁾	0.23 $\pm$ 0.02**	1.5
	+	6-AN-treated, starved ²⁾	0.83 $\pm$ 0.26*	5.5
	+	6-AN-treated, starved+SKF 525-A	N.D.	<0.1

The initial concentration of EPN added here was 1.5×10^{-5} M. The concentrations of cytosol and SKF 525-A used were 6–9 mg protein in 1 ml of incubation mixture and 1 mM, respectively. ¹⁾Rats were starved for 16 hr prior to sacrifice, and cytosol was prepared. ²⁾6-Aminonicotinamide (6-AN) was administered intraperitoneally to rats at a dose of 15 mg/kg 16 hr before sacrifice. Each value is the mean $\pm$ S.E. from 3 experiments. N.D.: less than 0.13×10^{-7} M. * $P < 0.05$ and ** $P < 0.01$ vs. the non-treated, fed-group.

taining liver microsomes and EPN without added NADPH-generating system.

As shown in Table 4, in vitro formation of EPN=O from EPN was significantly decreased by addition of liver cytosol from starved rats, as compared to that from fed rats, to liver microsomes from fed rats. On the other hand, addition of cytosol from 6-AN treated and starved rats stimulated EPN=O formation. These phenomena occurred to a lesser extent when liver microsomes of starved rats were used. In addition, in vitro inhibition of EPN=O formation by SKF 525-A supported the well known finding that EPN=O formation from EPN is catalyzed through cytochrome P-450-coupled drug metabolism in the liver.

Discussion

It has been well established that pure organophosphorothioates have virtually no activity against carboxylesterase and acetylcholinesterase in vitro; however, they become active in in vivo and in vitro studies by conversion to their respective oxygen analogs through the cytochrome P-450-coupled monooxygenase system in the presence of NADPH.

The present study demonstrates that NADPH content in liver cytosol was increased by treatment of rats with 6-AN, and this

seemed to be responsible for the potentiation of the inhibition of carboxylesterase by EPN. Thus, formation of EPN=O was increased by addition of the cytosol from 6-AN-treated rat liver. In addition, aminopyrine N-demethylase activity was also increased by addition of liver cytosol, instead of the NADPH-generating system, from fed rats which contained a high level of NADPH. On the other hand, Johnson and McColl (17) and Dietrich et al. (18) have reported that 6-AN can replace the nicotinamide moiety of NAD(P), giving rise to the formation of an abnormal NAD molecule. Therefore, we are tentatively assuming that the increase in NADPH in liver cytosol was due to the effect of 6-ANAD(P), but not 6-AN itself, formed in 6-AN-treated rat liver, because there was no significant increase in the anti-carboxylesterase action of EPN when 6-AN was added in the incubation mixture in vitro (data not shown).

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