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EFFECTS OF MIXED-FUNCTION OXIDASE MODIFIERS ON NEUROTOXICITY OF ACRYLAMIDE IN RATS

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Abstract—The effects of modifiers of the microsomal mixed-function oxidase system on acrylamide-induced hind-limb paralysis were investigated in rats. Pretreatment of rats with phenobarbital, *trans*-stilbene oxide or dichloro diphenyl trichloroethane (DDT) resulted in an earlier onset and subsequent development of acrylamide-induced hind-limb paralysis than that observed in animals treated only with acrylamide. Cobalt chloride pretreatment of rats caused a significant delay in the onset and development of hind-limb paralysis. Our results suggest that an intermediate formed by the cytochrome P-450 system may be responsible for acrylamide neurotoxicity.

Acrylamide ($CH_2 = CHCONH_2$) is used extensively as a monomer in the plastics and polymer industry [1]. Several cases of human poisoning with acrylamide have been reported both in occupational and non-occupational situations [1, 2]. Experimental studies have established that acrylamide is a potent neurotoxin that causes distal axonopathy of the "dying-back" type in the central and peripheral nervous systems [1, 2].

The metabolic fate of acrylamide and the factors that lead to its neurotoxic effects are not well understood, nor is it known whether acrylamide or its metabolite is responsible for its neurotoxic effects. Acrylamide has been shown to form glutathione-*S*-conjugates [3]. Our studies have established that conjugation of acrylamide with glutathione occurs both nonenzymically and enzymically, the latter being catalyzed by glutathione-*S*-transferases of rat liver and brain [4, 5]. The role of microsomal cytochrome P-450-dependent mixed-function oxidases in the biotransformation of acrylamide was postulated by Kaplan *et al.* [6]. Their suggestion was based on the increase and decrease in the single exposure toxicity of acrylamide in rats pretreated with SKF-525A and phenobarbital respectively. Recently Ortiz *et al.* [7] demonstrated the formation of a reactive microsomal metabolite of acrylamide under *in vitro* conditions. This metabolite was also found to be inhibitory to one form of aniline hydroxylase activity. We reasoned that, if an intermediate of acrylamide formed by the cytochrome P-450-dependent mixed-function oxidase system were responsible for the toxicity of acrylamide, then its toxicity would be altered in animals pretreated with mixed-function oxidase modifiers. We explored this hypothesis *in vivo* by using inducers and a depletor of the cytochrome P-450-dependent system. Our results suggest the involvement of a metabolite of acrylamide, at least in part, in the toxicity of the parent compound.

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MATERIALS AND METHODS

Animals and treatment. Male Wistar albino rats (90–110 g), obtained from the ITRC animal breeding colony and raised on a commercial pellet diet (Hindustan Lever, Bombay) and water *ad lib.*, were used in the present study. The animals were divided into eight groups of ten to twelve rats each and treated as follows.

Prior to acrylamide treatment, the animals of group IV to group VIII were pretreated for 2 days with daily injections of mixed-function oxidase modifiers to ensure the induced and inhibited status of the animals. The treatment of animals at the doses indicated below is optimal for achieving induction of the cytochrome P-450 system [8]. During the entire period of experiments, the animals in group IV to VIII were treated daily with intraperitoneal or subcutaneous injections of the mixed-function oxidase modifiers 2 hr before the treatment with acrylamide.
Group I: Control, injected i.p. with 0.15 M NaCl.
Group II: Control, injected i.p. with 0.2 ml of ground nut oil.
Group III: Acrylamide (50 mg/kg), injected i.p. in 0.2 ml of 0.15 M NaCl.
Group IV: Phenobarbital (50 mg/kg), injected i.p. in 0.2 ml of 0.15 M NaCl 120 min before acrylamide (50 mg/kg).
Group V: β-Naphthoflavone (20 mg/kg), injected i.p. in 0.2 ml of ground nut oil 120 min before acrylamide (50 mg/kg).
Group VI: *Trans*-stilbene oxide (200 mg/kg) in 0.2 ml of ground nut oil 120 min before acrylamide (50 mg/kg).

Group VII: Dichloro diphenyl trichloroethane (DDT) (12 mg/kg), injected i.p. in 0.2 ml of 0.15 M NaCl 120 min before acrylamide (50 mg/kg).
Group VIII: $CoCl_2$ (23 mg/kg), injected subcutaneously in 0.2 ml of 0.15 M NaCl 120 min before acrylamide (50 mg/kg).

Preparation of enzyme source from rat liver. The animals were killed by cervical dislocation. Livers were removed, blotted free of blood, and washed with ice-cold saline. Tissues were homogenized in 4 vol. of ice-cold 0.1 M phosphate buffer, pH 7.4, containing 0.15 M KCl. The liver homogenates were centrifuged at 9000 *g* for 20 min at 0°. The resulting supernatant fractions were subsequently centrifuged at 104,000 *g* for 1 hr in an MSE superspeed ultracentrifuge to obtain microsomes and cytosol. Microsomes were suspended in homogenizing buffer and used for the estimation of the cytochrome P-450 concentrations and activities of aminopyrine-*N*-demethylase and aniline hydroxylase. Liver cytosol was used for the measurement of the activity of glutathione-*S*-transferase.

Estimation of cytochrome P-450. The cytochrome P-450 contents were determined spectrophotometrically as described by Omura and Sato [9] from the carbon monoxide plus dithionite minus carbon monoxide difference spectra using a Cary 219 spectrophotometer. An extinction coefficient of 91 $mM^{-1}cm^{-1}$ was used for the absorbance change between 450 and 490 nm to determine the cytochrome P-450 concentration.

Enzyme assay. Aminopyrine-*N*-demethylase activity was assayed according to Cochin and Axelrod [10] by measuring the formation of formaldehyde according to Nash [11]. Aniline hydroxylase activity was assayed by measuring the formation of *p*-aminophenol [12]. Glutathione-*S*-transferase activity was determined according to the method of Habig *et al.* [13] using 1-chloro-2,4-dinitrobenzene as a substrate. Protein was determined according to Lowry *et al.* [14], using bovine serum albumin as a reference standard.

Assessment of hind-limb paralysis. The development of hind-limb paralysis in experimental animals was assessed as described earlier [15]. The paralysis test was performed three times a day at 8:00 a.m., 1:00 p.m. and 6:00 p.m. by different personnel on a double-blind basis to ensure a total lack of bias. The test reflects an all-or-none response. A Mann–Whitney U-test was employed to determine whether the modifiers altered the time of onset of paralysis.

RESULTS

Effects of inducers and depletor of microsomal mixed-function oxidases on acrylamide-induced hind-limb paralysis. The onset and subsequent development of acrylamide-induced hind-limb paralysis in control rats and in animals pretreated with modifiers of mixed function oxidases are shown in Fig. 1. The rats exposed to acrylamide showed progressive development of signs of neurotoxicity such as ataxia, tremors, and hind-limb paralysis. Following exposure to acrylamide 30, 60 and 100% of the animals exhibited signs of complete hind-limb paralysis on days 5, 6 and 8, respectively, of the treatment.

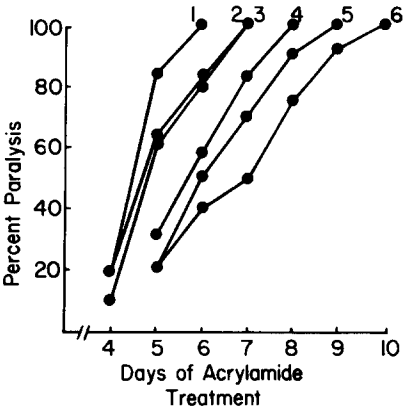

Fig. 1. Effects of phenobarbital, β-naphthoflavone, *trans*-stilbene oxide, DDT and $CoCl_2$ pretreatments of acrylamide-induced hind-limb paralysis in rats. Key: (1) phenobarbital + acrylamide; (2) *trans*-stilbene oxide + acrylamide; (3) DDT + acrylamide; (4) only acrylamide; (5) β-naphthoflavone + acrylamide; and (6) $CoCl_2$ + acrylamide. Other details are provided in the text.

Table 1. Mixed-function oxidase and glutathione-*S*-transferase activities and cytochrome P-450 concentrations in livers of variously treated animals

Treatment	Cytochrome P-450 (nmoles/mg protein)	Aminopyrine *N*-demethylase (nmoles formaldehyde/min/mg protein)	Aniline hydroxylase (nmoles *p*-aminophenol/min/mg protein)	Glutathione-*S*-transferase (nmoles CDNB conjugate/min/mg protein)
Normal	0.80 ± 0.11*	1.87 ± 0.05*	0.32 ± 0.02*	770 ± 30*
ACR	0.46 ± 0.04†	1.38 ± 0.03†	0.23 ± 0.04†	904 ± 23†
PB + ACR	1.72 ± 0.09†	ND‡	ND	1221 ± 23†
TSO + ACR	1.08 ± 0.03†	2.36 ± 0.06†	0.36 ± 0.03†	1512 ± 38†
DDT + ACR	1.77 ± 0.11†	2.52 ± 0.19†	0.42 ± 0.03†	1268 ± 20†
$CoCl_2$ + ACR	0.21 ± 0.02†	1.19 ± 0.04†	0.15 ± 0.04†	782 ± 27

* Each value is the mean ± S.E. of four animals. Saline- and ground nut oil-treated values did not differ and, therefore, they have been grouped together. Abbreviations: ACR, acrylamide; PB, phenobarbital; TSO, *trans*-stilbene oxide; and CDNB, 1-chloro-2,4-dinitrobenzene. For treatment and other details see text.
† Statistically significant difference from controls ($P < 0.05$) and from only acrylamide-treated group ($P < 0.05$).
‡ Not determined.

Pretreatment with phenobarbital, a cytochrome P-450 type of inducer, to rats resulted in an earlier onset and subsequent development of hind-limb paralysis than that observed in saline-treated animals. Eighteen and ninety percent of the animals were found to be paralyzed on days 4 and 5, respectively, of acrylamide exposure. Pretreatment of rats with the other cytochrome P-450 inducers, DDT and *trans*-stilbene oxide, also resulted in the early onset of hind-limb paralysis. Fifteen, sixty and eighty percent of the DDT- or *trans*-stilbene oxide-treated animals exhibited signs of complete hind-limb paralysis on days 4, 5 and 6, respectively, of acrylamide exposure.

Pretreatment of rats with β-naphthoflavone, a cytochrome P-448 type of inducer, caused no significant (Mann–Whitney U-Test) alteration in the development of acrylamide-induced hind-limb paralysis when compared to only acrylamide-treated rats (Fig. 1). Fifty, seventy and ninety percent of the animals exhibited signs of complete hind-limb paralysis on days 6, 7, and 8, respectively, on exposure to acrylamide.

In rats treated only with acrylamide, 100% of the animals were found to be paralyzed after 8 days of treatment. In phenobarbital-, DDT- and *trans*-stilbene oxide-pretreated rats, paralysis in 100% of the rats occurred on days 6, 7 and 7, respectively, on exposure to acrylamide. In β-naphthoflavone-pretreated rats, 100% of the animals were paralyzed on day 9 of acrylamide exposure.

Pretreatment of rats with cobalt chloride, a depletor of cytochrome P-450, resulted in the delayed onset of hind-limb paralysis. Forty, fifty, seventy-five, ninety and one hundred percent of the animals exhibited signs of complete hind-limb paralysis on days 6, 7, 8, 9 and 10, respectively, of acrylamide exposure.

Effects of inducers and depletor of microsomal mixed-function oxidases on cytochrome P-450 contents and enzyme activities in acrylamide-exposed rats. Data in Table 1 show the effects of modifiers of mixed-function oxidases on the levels of hepatic microsomal cytochrome P-450 and the activities of aniline hydroxylase, aminopyrine-*N*-demethylase and cytosolic glutathione-*S*-transferase, in control, acrylamide- and variously pretreated rats following 7 days of acrylamide exposure. At this time period of acrylamide exposure, 80% of the animals in the acrylamide-treated group were paralyzed. One hundred percent of the phenobarbital-, *trans*-stilbene oxide- or DDT-pretreated groups and 50% of the $CoCl_2$-pretreated group were found to be paralyzed at the same time period of acrylamide exposure.

Four animals, exhibiting signs of complete hind-limb paralysis, from each group were killed for the measurement of microsomal cytochrome P-450 levels and aniline hydroxylase, aminopyrine-*N*-demethylase and cytosolic glutathione-*S*-transferase activities. These measurements were made to ensure that the pretreatment regimen used for the present study was adequate for the induction and inhibition of mixed-function oxidases. These results are consistent with the earlier observations [16, 17] that exposure of rats to acrylamide causes a decrease in the levels of cytochrome P-450 and aminopyrine-*N*-demethylase and aniline hydroxylase enzyme activities. Animals receiving the combined treatment of acrylamide and $CoCl_2$ exhibited a greater decrease in the levels of mixed-function oxidases. Administration of acrylamide to phenobarbital, *trans*-stilbene oxide- or DDT-pretreated rats resulted in 115, 35 and 121% increases, respectively, in hepatic cytochrome P-450 concentrations.

DISCUSSION

Ortiz *et al.* [7] have demonstrated recently that the *in vitro* biotransformation of acrylamide by liver microsomes from untreated and phenobarbital-treated rats results in the formation of a reactive intermediate that is inhibitory to one form of rat liver microsomal aniline hydroxylase. Although the identity of the presumed electrophilic intermediate of acrylamide was not ascertained by these investigators, it was established that no reactive intermediate was formed in the absence of oxygen or NADPH. The possibility of the formation of an intermediate has also been suggested by the studies of Agrawal *et al.* [18] which demonstrated that the pretreatment of rats with SKF-525A completely prevents the acrylamide-induced changes of striatal dopaminergic receptor activity. However, studies conducted a decade ago do not suggest the involvement of a metabolite in acrylamide toxicity as pretreatment of hens [19] with phenobarbital caused no change in the development of peripheral acrylamide neuropathy. In the latter study, the induced status of mixed-function oxidase was not ascertained.

It can also be argued that the metabolite of acrylamide formed by the cytochrome P-450 system represents a detoxification mechanism. According to this argument, it would be expected that in induced animals more of the acrylamide would be detoxified which, in turn, would be expected to result in the delayed toxicity of acrylamide. The argument is not supported by our observations that the pretreatment of rats with inducers of cytochrome P-450 resulted in early acrylamide toxicity. Similarly, our results show that the pretreatment of rats with a depletor of cytochrome P-450, $CoCl_2$, resulted in delayed acrylamide toxicity.

Our hypothesis that a "presumed" intermediate of acrylamide formed by the cytochrome P-450 system is responsible for the toxicity of acrylamide is supported by the earlier appearance of neurotoxicity in rats treated with the inducers of cytochrome P-450. The delayed development of acrylamide toxicity in $CoCl_2$-pretreated animals further supports the concept that the observed toxicity of acrylamide is, at least in part, mediated by an intermediate formed by the mixed-function oxidase system. Failure of β-naphthoflavone to modify the development of acrylamide-induced hind-limb paralysis suggests that the possible intermediate of acrylamide is formed by the cytochrome P-450 system rather than by the cytochrome P-448 system.

In summary, our studies strongly suggest that a metabolic intermediate of acrylamide formed by the cytochrome P-450-dependent system is responsible, at least in part, for the observed neurotoxicity of acrylamide.

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REFERENCES

1. P. S. Spencer and H. H. Schaumberg, *Can. J. neurol Sci.* **1**, 143 (1974).
2. *NIOSH Criteria for a Recommended Occupational Exposure to Acrylamide.* U.S. Department of Health, Education and Welfare, Washington, DC (October 1976).
3. P. M. Edwards, *Biochem. Pharmac.* **24**, 1277 (1975).
4. R. Dixit, H. Mukhtar, P. K. Seth and C. R. Krishna Murti, *Chem. Biol. Interact.* **32**, 353 (1980).
5. R. Dixit, H. Mukhtar, P. K. Seth and C. R. Krishna Murti, *Biochem. Pharmac.* **30**, 1739 (1981).
6. M. L. Kaplan, S. D. Murphy and F. H. Gilies, *Toxic. appl. Pharmac.* **24**, 564 (1973).
7. E. Ortiz, J. M. Patel and K. C. Liebman, *Adv. exp. Med. Biol.* **136**, 1221 (1981).
8. H. Mukhtar, T. H. Elmamlouk and J. R. Bend, *Chem. Biol. Interact.* **22**, 125 (1978).
9. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
10. J. Cochin and J. Axelrod, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
11. T. Nash, *Biochem. J.* **55**, 416 (1953).
12. R. Kato and J. R. Gillette, *J. Pharmac. exp. Ther.* **150**, 279 (1965).
13. W. H. Habig, M. J. Pabst and W. B. Jakoby, *J. biol. Chem.* **249**, 7130 (1973).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. R. Dixit, R. Husain, P. K. Seth and H. Mukhtar, *Toxic. Lett.* **6**, 417 (1980).
16. O. G. Nilsen, R. Toftgard, M. I. Sundberg and J. A. Gustafsson, *Acta pharmac. tox.* **43**, 299 (1978).
17. M. Das, H. Mukhtar and P. K. Seth, *Toxic. appl. Pharmac.* **66**, 420 (1982).
18. A. K. Agrawal, P. K. Seth, R. E. Squibb, H. A. Tilson, L. L. Uphouse and S. C. Bondy, *Pharmac. Biochem. Behav.* **14**, 527 (1981).
19. P. M. Edwards, *Br. J. ind. Med.* **32**, 31 (1975).