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Cypermethrin-induced oxidative stress in rat brain and liver is prevented by Vitamin E or allopurinol

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Abstract

Considering that the involvement of reactive oxygen species (ROS) has been implicated in the toxicity of various pesticides, this study was designed to investigate the possibility of oxidative stress induction by cypermethrin, a Type II pyrethroid. Either single (170 mg/kg) or repeated (75 mg/kg per day for 5 days) oral administration of cypermethrin was found to produce significant oxidative stress in cerebral and hepatic tissues of rats, as was evident by the elevation of the level of thiobarbituric acid reactive substances (TBARS) in both tissues, either 4 or 24 h after treatment. Much higher changes were observed in liver, increasing from a level of 60% at 4 h up to nearly 4 times the control at 24 h for single dose. Reduced levels (up to 20%) of total glutathione (total GSH), and elevation of conjugated dienes ($\sim$60% in liver by single dose at 4 h) also indicated the presence of an oxidative insult. Glutathione-S-transferase (GST) activity, however, did not differ from control values for any dose or at any time point in cerebral and hepatic tissues. Pretreatment of rats with allopurinol (100 mg/kg, ip) or Vitamin E (100 mg/kg per day, ig, for 3 days and a dose of 40 mg/kg on the 4th day) provided significant protection against the elevation of TBARS levels in cerebral and hepatic tissues, induced by single high dose of oral cypermethrin administration within 4 h. Thus, the results suggest that cypermethrin exposure of rats results in free radical-mediated tissue damage, as indicated by elevated cerebral and hepatic lipid peroxidation, which was prevented by allopurinol and Vitamin E.

Keywords: Allopurinol; Cypermethrin; Oxidative stress; Vitamin E

1. Introduction

The synthetic pyrethroids constitute an unique group of insecticides having pyrethrin-like structures with better performance characteristics and account for over 30% of insecticide use globally (Vijverberg and van den Bercken, 1982; Soderlund and Bloomquist, 1989). The pyrethroids are potent neurotoxicants in both vertebrates and invertebrates, but acute toxicity in mammals is low (Elliot and Janes, 1978). Their selective toxicity seems to be based mainly on the responses of neuronal sodium channels, and partly on metabolic degradation (Narahashi, 1996). On the

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basis of different behavioral, neurophysiological and biochemical profiles, two distinct classes of pyrethroids have been identified. Type I pyrethroids may cause mainly hyperexcitation and fine tremors; while Type II pyrethroids possess a α cyano group and produce a more complex syndrome, including clonic seizures (Vershoyle and Aldridge, 1980). However, the signs of toxicity disappear fairly rapidly, and animals recover, generally, within a week.

Pyrethroids are more hydrophobic than other classes of insecticides (Michelangeli et al., 1990) and this feature indicates that the site of action is in the biological membrane. In fact, the principal target site for pyrethroids is defined as the voltage-dependent sodium channel in the neuronal membrane (Narahashi, 1985; Soderlund and Bloomquist, 1989; Vijverberg and van den Bercken, 1990). The available data indicate that both Type I and Type II pyrethroids act potently and stereoselectively on sodium channels by slowing kinetics of both opening and closing of individual channels. Inhibition of $GABA_A$ receptor is an additional mechanism proposed for Type II pyrethroids (Narahashi, 1991).

It was of interest to investigate the possibility of oxidative stress induction by pyrethroids, considering the above mentioned data and taking the followings into account. (1) There is evidence that excitatory events may stimulate reactive oxygen species (ROS) production, but there is also evidence for a reciprocal relation between the two phenomena. ROS in central nervous system (CNS) elevate free intracellular calcium release; increase the release of excitatory amino acids, mainly glutamate; cause lipid peroxidation and depletes neuronal glutathione (GSH) (Bondy and Lebel, 1993). (2) The involvement of ROS in the toxicity of various pesticides has been reported, including chlorinated hydrocarbon endosulfan (Hincal et al., 1995) and organophosphates (Yang et al., 1996). The induction of oxidative stress and alteration of antioxidant system by pyrethroids in rats have been reported recently by Gupta et al. (1999), Kale et al. (1999a,b). However some of their findings were rather controversial. In order to better understand the involvement of oxidative stress in the pyrethroid action, we investigated the oxidative stress inducing effects of a Type II pyrethroid, cypermethrin [(RS) α-cyano-3-phenoxybenzyl (1RS)-*cis*-*trans*-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane carboxylate] in rats.

2. Materials and methods

2.1. Chemicals

Technical grade cypermethrin was from Novartis (Istanbul, Turkey) and had a purity of $>91\%$. Vitamin E (α-tocopherol); allopurinol; NADPH; oxidized glutathione (GSSG) and reduced GSH; 1-chloro-2,4 dinitrobenzene; glutathione reductase; 2-thiobarbituric acid; *N*-ethylmaleimide; 5,5′-dithiobis-(2-nitrobenzoic acid); and bovine serum albumin (BSA) were obtained from Sigma (St Louis, MO, USA). All the other reagents used were of analytical reagent grade and obtained either from Merck (Darmstadt, Germany) or BDH (Poole, Dorset, UK).

2.2. Animals and treatments

Male albino Wistar rats, weighing 180 ± 20 g were obtained from Animal Care Unit of Hacettepe University, and were maintained on a 12-h light:12-h dark cycle in plastic cages. They were provided with standard laboratory chow and allowed free access to food and water. Cypermethrin was administered in (1 ml) corn oil by oral (ig) route, and control animals received a corresponding amount of corn oil in an identical manner. The single dose cypermethrin group received 170 mg/kg cypermethrin ($\sim 2/3$ LD_{50}) and were decapitated 4 or 24 h later. Repeated dosing was carried out at 75 mg/kg per day dose ($\sim 1/3$ LD_{50}), for 5 days and animals were decapitated 24 h after the last dose of cypermetrin.

As the second part of the experiment, two groups of animals were pretreated, either with Vitamin E or allopurinol as described, below, along with appropriate vehicle controls.

2.2.1. Treatment with Vitamin E

Rats were pretreated with a loading dose of 100 mg/kg per day, ig, Vitamin E in corn oil for 3

days, and then received a single maintenance dose of 40 mg/kg on the 4th day. Fifteen minutes later, animals received 170 mg/kg, ig, cypermethrin and were decapitated four hours later.

2.2.2. Treatment with allopurinol

Rats received a single dose of 100 mg/kg, ip, allopurinol (in 1 ml distilled water, adjusted to pH 11 with 1 N NaOH), and 15 min later, 170 mg/kg, ig, cypermethrin was administered. Four h later animals were decapitated.

2.3. Preparation of tissue homogenates and biochemical assays

After decapitation, whole brain (cerebrum, cerebellum and brain stem) and liver were rapidly removed and kept on ice. The homogenization was immediately carried out in a Teflon-glass homogenizer in a volume of ice-cold buffer containing 140 mM KCl, 10 mM $NaHCO_3$, 3mM KH_2PO_3 and 2 mM K_2HPO_4 (adjusted to pH 7.2) to obtain a 10% (w/v) whole homogenate. All biochemical parameters were determined immediately on the same day. The concentration of thiobarbituric acid reactive substances (TBARS) in whole homogenates of liver or cerebral tissues were determined, as a measure of lipid peroxidation according to the procedure of Ohkawa et al. (1979). Immediately after the tissues were homogenized, as described above, extraction and purification of total tissue lipids and conjugated dienes measurements were carried out by second derivative spectroscopy (Buege and Aust, 1978; Corongiu and Milia, 1983). The activity of glutathione-S-transferase (GST) in cytosolic fractions of tissues was determined in cytosolic fractions of tissues, using 1-chloro-2,4 dinitrobenzene as a substrate, according to the method of Habig et al. (1974). The total GSH content of the tissues were determined by an enzymatic cycling assay as described by Tietze (1969) and modified by Hazelton and Lang (1980). The protein concentrations were determined by the standard method of Lowry et al. (1951).

2.4. Statistics

The data were analyzed by one-way ANOVA and the significant differences between the controls and the treated groups were evaluated by Student's *t*-test. A P value of 0.05 was considered significant.

3. Results

There was no mortality in any groups at any point in time. Minor symptoms of neurotoxicity, such as abnormal gait and nervousness, were noted in only a small number of animals (3/12) treated with single high dose of cypermethrin.

3.1. Lipid peroxidation in cerebral and hepatic tissues of rats exposed to cypermethrin

Single or repeated oral administration of cypermethrin to rats caused enhanced lipid peroxidation in hepatic and cerebral tissues of rats, as indicated by increased TBARS levels (Table 1). The increase was significant at all measurement points, except in cerebral tissue 24 h after a single high dose. The highest elevation of TBARS in the brain was noted 4 h after the single high dose. However, in hepatic tissue, much higher changes were observed and the effect of the single high dose of cypermethrin was found to increase with time. The increase was up to near 4 times the control level for single dose at 24 h. Whereas the increase caused by repeated low dosing was lower than that caused by single high dosing at any point in time.

The level of conjugated dienes was measured as a second indicator of lipid peroxidation in single dose cypermethrin treated rats and found to be significantly increased in both tissues, when measured 4 h after a single high dose of cypermethrin administration (Fig. 1). The enhancement in liver was much higher ($\sim 60\%$) also.

3.2. Glutathione level

Total GSH levels measured in both tissues are shown in Table 2. Similarly, hepatic GSH levels were found to be diminished similarly after single and repeated dosing. Cerebral GSH content was

Table 1
TBARS levels measured in cerebral and hepatic tissues of rats treated with, ig, cypermethrin[a]

Tissue	Control group ($n = 20$)[b]	Cypermethrin treated group				
		Cypermethrin 75 mg/kg per day for 5 days ($n = 5$)[c]	Cypermethrin (24 h) 170 mg/kg ($n = 5$)[c]	Cypermethrin (4 h) 170 mg/kg ($n = 6$)[d]	Cypermethrin (4 h) + Allopurinol ($n = 6$)[e]	Cypermethrin (4 h) + Vitamin E ($n = 6$)[f]
Cerebral	1.88 ± 0.04	2.11 ± 0.05**	1.85 ± 0.08	2.55 ± 0.05*	1.75 ± 0.05**	1.76 ± 0.05**
Hepatic	2.13 ± 0.06	2.85 ± 0.04*	8.22 ± 1.00*	3.42 ± 0.33*	2.23 ± 0.04**	1.98 ± 0.06**

[a] Values are given as nmol TBARS/mg protein (mean ± SEM).

[b] Three groups ($n = 4$) of vehicle (corn oil) control and two groups ($n = 4$) of pretreatment plus vehicle control experiments were performed parallel to each treatment group. Mean value of all the groups was used as the overall control value, since there were no significant differences among groups.

[c] Animals were decapitated 24 h after the last dose of cypermethrin.

[d] Animals were decapitated 4 h after the cypermethrin dose.

[e] Animals received 100 mg/kg, ip, allopurinol and 15 min later 170 mg/kg cypermethrin, and were decapitated 4 h later.

[f] Animals were pretreated with 100 mg/kg per day, ig, Vitamin E for 3 days and with a dose of 40 mg/kg on the 4th day. Fifteen minutes later, they received 170 mg/kg cypermethrin and were decapitated 4 h later.

* $P < 0.05$ versus control group.

** $P < 0.05$ versus single dose cypermethrin (4 h).

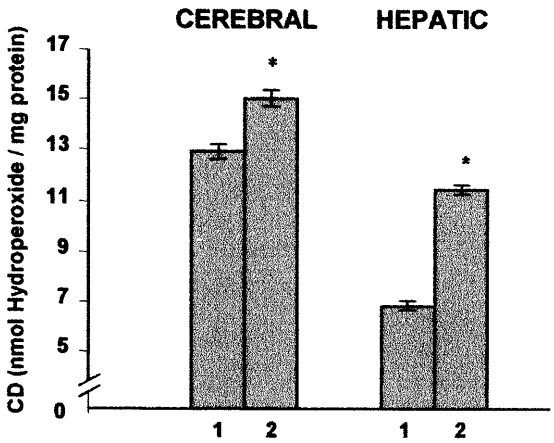

Fig. 1. Conjugated diene (CD) levels in cerebral and hepatic tissues of rats treated with a single dose of ig, cypermethrin. The treatment groups are indicated by: (1) Control groups ($n = 6$), (2) Single dose cypermethrin, 170 mg/kg ($n = 6$). Animals were decapitated 4 h after the cypermethrin dose or vehicle. Values are given as mean ± SEM. $*P < 0.5$ versus control.

also diminished, when exposed to cypermethrin, but the effect of a single cypermethrin dose did not last for 24 h.

3.3. Glutathione-S-transferase activity

The GST activities measured in cerebral and hepatic tissues did not differ from the control values using any of the dose or at any time point (Table 2).

3.4. Allopurinol or Vitamin E pretreatment

Pretreatment of rats with allopurinol or Vitamin E provided significant protection against the elevation of the TBARS levels in cerebral and hepatic tissues induced by single high dose of oral cypermethrin within 4 h of administration (Table 1).

4. Discussion

The overall results of this study showed that oral exposure to cypermethrin introduces significant oxidative stress in cerebral and hepatic tissues of rats as was evident by the elevation of the level of TBARS and conjugated dienes. Reduced level of total GSH also indicated the occurrence of an oxidative insult. This study is the first to show that the xanthine oxidase (XO) inhibitor and radical scavenger allopurinol, and the chain breaking antioxidant, Vitamin E, can prevent cypermethrin-induced lipid peroxidation in rats.

Although GSSG levels were not measured in the present study, a small but significant total GSH depletion was observed both with single and

Table 2
Total GSH levels and GST activity in cerebral and hepatic tissues of rats treated with, ig, cypermethrin[a]

Tissue	Parameters	Control group ($n = 12$)[d]	Cypermethrin treated group		
			Cypermethrin 75 mg/kg per day for 5 days ($n = 6$) [e]	Cypermethrin (24 h) 170 mg/kg ($n = 6$) [e]	Cypermethrin (4 h) 170 mg/kg ($n = 6$)[f]
Cerebral	Total GSH[b]	1.92 ± 0.05	1.75 ± 0.06 *	2.01 ± 0.04	1.73 ± 0.03*
	GST[c]	0.15 ± 0.10	0.16 ± 0.004	0.15 ± 0.02	0.17 ± 0.04
Hepatic	Total GSH[b]	8.85 ± 0.44	7.19 ± 0.11*	7.23 ± 0.44*	7.50 ± 0.14*
	GST[c]	1.10 ± 0.01	1.22 ± 0.11	1.00 ± 0.09	1.17 ± 0.05

[a] Values are given as mean ± SEM.
[b] μmol GSH equivalent/g tissue (wet weight).
[c] μmol/min per mg protein.
[d] Three groups ($n = 4$) of vehicle (corn oil) control experiments were performed parallel to each treatment groups. Mean value of all the groups was used as the overall control value, since there were no significant differences between groups.
[e] Animals were decapitated 24 h after the last dose of cypermethrin.
[f] Animals were decapitated 4 h after the cypermethrin dose.
* $P < 0.05$ versus control group.

repeated doses of cypermethrin in hepatic tissue of rats at all time points. A total GSH depletion was also observed in cerebral tissue, but the degree of decrease was lower. It appeared that single high dose of cypermethrin initiated an immediate effect and increased the oxidatively damaged end-products of lipids in cerebral tissue as was observed by high levels of TBARS and conjugated dienes, and the diminution of total GSH content at 4 h. But, this effect did not last long and relatively decreased levels of TBARS and increased levels of total GSH were found at 24 h. Whereas with the repeated dosing, it was possible to observe a small but significantly high lipid peroxidation (TBARS levels) even after 24 h of the last dose. These results may suggest the possibility of a redistribution occurring following a rapid initial penetration of highly lipophilic cypermethrin into the CNS. However, lipid peroxidation was much higher in liver; the highest elevation of TBARS was noted 24 h after a single high dose, suggesting the production of oxidative metabolites and/or free radicals possibly continues during the intensive hepatic metabolism, and this may also be due to the progressive nature of the free radical chain reactions.

GST detoxifies a variety of electrophilic compounds to less toxic forms by conjugation with -SH groups, such as GSH; and also reduces lipid peroxides (Mosialou et al., 1993). In the present study, GST activity was not changed by any of the exposure schemes at any point in time, neither in hepatic nor cerebral tissues. In fact, earlier studies indicated only a slight induction of microsomal enzymes by pyrethroids (Riviere et al., 1983), and cypermethrin did not exert any significant effect on cytosolic GST when administered orally in 80 mg/kg per day (0.25 LD_{50}) doses for up to 10 days in rats (Krechniak and Wrzesniowska, 1991). In contrast, Kale et al. (1999a,b) reported recently a significant decrease in GST activity, and $>50\%$ of enhancement of GSH in rat livers after 24 h of the administration of a single oral dose as low as 0.001% LD_{50} of cypermethrin. The decrease in GST activity continued up to 3 days and only a partial recovery was observed on the day 14; while a complete recovery was noted for the GSH content by that time. They also observed marked TBARS elevations in various tissues, including erythrocytes. The level of TBARS in all tissues was highest on the day 1, and returned to the control level on the day 14. However, the results of Gupta et al. (1999) are in accordance with our findings; along with lipid peroxidation in various tissues, they observed $\sim 15\%$ decrease in GSH content of brain in neonatal rats exposed to an allethrin containing repellant by inhalation for 8 days. The explanation or meaning of these conflicting results is not clear. Differences in dosage levels, and animal strains and ages might be the first factors to be considered. In addition, on the basis of our observations, a dose as low as 0.001% LD_{50} of cypermethrin, does not seem to induce such a level of oxidant stress and alteration of antioxidant system in the rat. Although we are not able to present a complete dose-dependency data, the doses used in this investigation were chosen after a preliminary screening. Hence, with doses lower than 20% LD_{50} of cypermethrin, no lipid peroxidation was observed.

The effectiveness of allopurinol against cypermethrin-induced oxidative stress might suggest, first, that XO be involved in cypermethrin-induced generation of free radicals. Although the biotransformation data of cypermethrin as referred in the literature do not present a specific involvement of XO, it is possible that free radicals may occur during the oxidative metabolism of cypermethrin. The generated free radicals might cause the degradation of purine nucleotides in the tissue and trigger XO-mediated superoxide generation. The effect of allopurinol pretreatment might, thus, be through the prevention of this second phase by inhibiting XO activity. But, due to the limitations of the presented data, such a possibility cannot be differentiated.

Another possibility for the observed protective effect of allopurinol is a direct antioxidant effect. In fact, in addition to its ability to inhibit XO, allopurinol is known to act, dependent on the dose, as a free radical scavenger and hence an antioxidant (Das et al., 1987; Augustin et al.,

1996b). Moorhouse et al. (1987) previously showed that allopurinol is a scavenger of highly reactive hydroxyl radicals, but to effectively scavenge oxygen radicals its concentration must be ≥ 500 μM. Augustin et al. (1996a,b) reported that the allopurinol concentrations in aqueous humor and retinal tissues showed, a dose dependency reaching scavenger concentrations after application of 50 mg/kg; or when given at a scavenger dose (500–1000 μmol). Lower doses (< 100 μmol), which inhibit XO activity effectively, showed no effect on oxidative tissue damage, as was evident by unchanged TBARS levels and GSSG/GSH ratio. Klein et al. (1996), also found that while 2–50 mg/kg of oral allopurinol effectively suppresses XO activity in the rat liver and intestine, antioxidant activity did not occur until doses of 100 mg/kg were administered. Considering the high ip dose applied to rats in the present study, it is possible to say, therefore, that the observed protective effect of allopurinol pretreatment is the result of its direct antioxidant and radical scavenger activity.

The preventive effect of Vitamin E against cypermethrin-induced elevation of TBARS in rat tissues, observed in this study, is in agreement with its antioxidant activity, and might also be relevant to its prophylactic and therapeutic effect on pyrethroid-induced abnormal skin sensation or paraesthesia in humans (Tucker et al., 1984). Skin paraesthesia is known to occur due to repetitive firing of sensory nerve endings (Vijverberg and van den Bercken, 1990). Patch clamp experiments performed by Song and Narahashi (1995) demonstrated that Vitamin E selectively blocks the pyrethroid-modified sodium channel in a dose dependent manner without affecting normal sodium channels. Although the mechanism of this effect was not clearly understood, the authors stated that it was unlikely that antioxidant or the free radical removing action of tocopherol played a role in reducing pyrethroids toxicity, because there was no evidence that pyrethroids acted as an oxidant or a free radical (Song and Narahashi, 1995; Narahashi, 1996). But, the results of the present study, along with the three recent publications (Gupta et al., 1999; Kale et al., 1999a,b) are in contrast to this conclusion. Therefore, it appears that in the actions of Vitamin E in pyrethroids intoxications, its antioxidant and membrane stabilizing effects are needed further consideration and elucidation. Furthermore, in cases of skin paraesthesia, a longer half life of the pyrethroids in plasma, and differences in individual levels of carboxylesterase activity in the skin were reported (Leng et al., 1999). Thus, in susceptible individuals, it is possible that the products of oxidative metabolites have a critical role.

In conclusion, the results of this study show that cypermethrin exposure of rats introduces significant oxidative stress in cerebral and hepatic tissues. However, it is certain that further studies are needed for better understanding of the causes of oxidative stress induction, as well as the mechanism of action and resulting toxicity symptoms of cypermethrin, and possibly of pyrethroids as a whole.

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