ORIGINAL PAPERS

PARTIAL PROTECTION FROM ORGANOPHOSPHATE-INDUCED CHOLINESTERASE INHIBITION BY METYRAPONE TREATMENT

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Abstract

Background: Organophosphates are cholinesterase (ChE) inhibitors with worldwide use as insecticides. Stress response, evidenced by a dramatic and relatively long-lasting (several hours) rise in the plasma glucocorticoid concentration is an integral element of the organophosphate (OP) poisoning symptomatology. In rodents, corticosterone (CORT) is the main glucocorticoid. There are several reports suggesting a relationship between the stressor-induced rise in CORT concentration (the CORT response) and the activity of the cerebral and peripheral ChE. Thus, it seems reasonable to presume that, in OP intoxication, the rise in plasma CORT concentration may somehow affect the magnitude of the OP-induced ChE inhibition. Metyrapone (MET) [2-methyl-1,2-di(pyridin-3-yl)propan-1-one] blocks CORT synthesis by inhibiting steroid 11β-hydroxylase, thereby preventing the CORT response. Chlorfenvinphos (CVP) [2-chloro-1-(2,4-dichlorophenyl) ethenyl diethyl phosphate] is an organophosphate insecticide still in use in some countries. **Material and Methods:** The purpose of the present work was to compare the CVP-induced effects – the rise of the plasma CORT concentration and the reduction in ChE activity – in MET-treated and MET-untreated rats. Chlorfenvinphos was administered once at 0.0, 0.5, 1.0 and 3.0 mg/kg *i.p.* Metyrapone, at 100 mg/kg *i.p.*, was administered five times, at 24-h intervals. The first MET dose was given two hours before CVP. **Conclusion:** The following was observed in the MET-treated rats: i) no rise in plasma CORT concentration after the CVP administration, ii) a reduced inhibition and a faster restitution of blood and brain ChE activities. The results suggest that MET treatment may confer significant protection against at least some effects of OP poisoning. The likely mechanism of the protective MET action has been discussed.

Key words:
Chlorfenvinphos, Metyrapone, Cholinesterase, Corticosterone

INTRODUCTION

The organophosphate (OP) compounds are one of the most common pesticides. Their high toxicity combined with the mode of use and easy accessibility result in a high number of intoxications, especially in developing countries [1–3]. Numerous human studies have shown that acute as well as repeated exposures to OP may produce long-lasting health consequences [4,5]. The basic mechanism of the OP toxic action – in target as well nontarget organisms, including man – consists in inhibition of acetylcholinesterase (AChE), an enzyme decomposing acetylcholine (ACh). Consequently, excessive ACh supply and

the resulting overstimulation of cholinergically innervated brain structures and peripheral effectors is regarded as the primary cause of the acute toxicity as well as the long-term effects of the OP exposure [6,7]. Functional imbalance in neurotransmitter systems triggers adaptive mechanisms. The well known adaptation to OP intoxication consists in down-regulation of cholinergic receptors [8,9]. Another one is the activation of AChE synthesis [10,11]. Those adaptive responses restore the balance in the cholinergic transmission.

A consistent symptom in OP intoxication is a strong activation of the hypothalamo-pituitary-adrenal (HPA) axis which is evidenced by a manifold and relatively long-lasting (up to ten or more hours) increase in the blood glucocorticoid concentration [12,13]. Treatment with atropine, a muscarinic antagonist, prevents this effect, thus suggesting that it may be related to the cholinergic overstimulation [14,15]. The rise in glucocorticoid concentration in OP intoxication deserves attention for several reasons. First, it confirms that OPs are powerful chemical stressors. Second, it is known that overproduction of glucocorticoids may result in morphological and functional changes in some brain structures [16,17] which allows one to suspect its relationship with at least some of the known long-lasting effects of OP exposure. And third, it is likely that glucocorticoids may somehow influence the adaptations related with the ChE activities. The main glucocorticoid in rodents is corticosterone (CORT). Blood CORT concentration rises after exposure to stressors. Numerous animal studies revealed marked changes in AChE activity, mainly in brain, after exposure to various nonchemical stressors. In some cases, however, the observed changes consisted in an increase [11,18–21], whereas in others in a decrease [22–24] of AChE activity. No changes were observed in adrenalectomized animals [18,20,25]. Treatment with CORT also results in changes in AChE activity [20,26,27], which suggests that the changes observed in stressed animals

were due to the CORT response, i.e. the stressor-induced rise of the CORT release from adrenals. An involvement of glucocorticoids in the control of AChE activity is also suggested by some *in vitro* studies; they showed a marked stimulation of AChE synthesis in P12 cells treated for 6 h with CORT [28]. The observations cited above allow one to presume that in the OP intoxication the magnitude and the dynamics of the AChE response may be somehow affected by the CORT response. The existing observations concerning this issue are scarce and inconsistent; some report no effect of adrenalectomy (i.e. a surgical blockade of CORT synthesis) on the level of the OP-induced AChE inhibition [15,25] whereas in others a slowing of the rate of AChE restitution was noted [29].

The purpose of the present experiment was to find out whether and in what way blocking the CORT response affects the level of inhibition and the restitution rate of ChE activity in blood (plasma and erythrocytes) and brain of rats intoxicated with an OP insecticide. The insecticide used was chlorfenvinphos [2-Chloro-1-(2,4-dichlorophenyl) ethenyl] diethyl phosphate)] – CVP, a compound used in our earlier studies. Chlorfenvinphos is a direct ChE inhibitor [30] and efficient inducer of the CORT response [13,31]. Metyrapone {[2-methyl-1,2-di(pyridin-3-yl)propan-1-one] – MET} was used for blocking the CORT response. Metyrapone blocks the CORT synthesis by inhibiting steroid 11β-hydroxylase [32].

MATERIALS AND METHODS

Animals

Adult male Wistar rats (325–340 g), outbreds, from our Institute's own breeding facility were used. Two weeks before the experiment onset they were transferred to the experimental facility where they were housed in polypropylene rat cages, one animal per cage. The temperature (22°C), humidity (50–60%) and the light/dark cycle (12/12 h with

Table 1. Experimental groups and treatment

Group name	Animals treated (n)	Dose (mg CVP/kg b.w.)	Cotreatment
SAL/0.0	20	0.0 (oil)	5×saline
SAL/0.5	20	0.5	5×saline
SAL/1.0	20	1.0	5×saline
SAL/3.0	20	3.0	5×saline
MET/0.0	20	0.0 (oil)	5×MET
MET/0.5	20	0.5	5×MET
MET/1.0	20	1.0	5×MET
MET/3.0	20	3.0	5×MET

light on from 6:00 a.m. to 6:00 p.m.) were controlled automatically. The cages and cage bedding (hardwood shavings) were changed twice a week. Food (Murigran pellets from AGROPOL, Motycz, Poland) and tap water were accessible *ad libitum*. Body weight was measured routinely once a week. At the beginning of the experiment the animals were divided into eight groups, 20 in each group (Table 1).

All animal use procedures were approved by the local Bio-ethical Committee (Medical University of Lodz, Poland, Decision No. 33/ŁB370/2007).

Chemicals and administration procedures

Chlorfenvinphos {[2-Chloro-1-(2,4-dichlorophenyl)ethenyl] diethyl phosphate – CVP}, technical grade, was obtained from the manufacturer (ORGANIKA-AZOT, Jaworzno, Poland). Metyrapone {[2-methyl-1,2-di(pyridin-3-yl)propan-1-one] – MET} was purchased from SIGMA. In order to obtain the required concentrations, CVP was diluted with olive oil and MET was dissolved in physiological saline (SAL). Both substances were administered intraperitoneally at 1.0 ml/kg b.w.

Chlorfenvinphos was administered once at doses of 0.0 (pure olive oil), 0.5, 1.0 or 3.0 mg/kg b.w. Metyrapone at daily doses of 100 mg/kg b.w., or SAL, were administered five times. The first MET injection was performed 2 h before the CVP injection and was repeated during the following four days at approximately the same hour (between 8:00 and 9:00 a.m.). The CVP doses were selected on the basis of data from our earlier studies on the same compound [14]. The daily MET dose was established on the basis of published reports [33,34], as well as our own unpublished observations.

Biochemical analyses

The animals (four per each time point) were killed by decapitation at selected time intervals, i.e. 3 h, 24 h, 7 days, 14 days and 21 days after the CVP administration. (In our earlier studies we have found that at the 3.0 mg/kg b.w CVP *i.p.* maximum ChE inhibition occurs about 3 h after the administration and the enzyme activity returns to normal level within about three weeks time [12]. The arteriovenous blood 5 ml samples were collected into Vacutainers containing an anticoagulant (K$_2$EDTA, 10.8 mg). After centrifuging (10 min, 1780×g), the plasma was transferred to Eppendorf vials and stored at –20°C until analysis. Erythrocyte 0.1 ml samples were mixed with 3.9 ml of distilled water and stored at –20°C until analysis.

The brains were removed from skulls as quickly as possible, chilled in ice-cold physiological saline and stored at –20°C until analysis.

Serum corticosterone assays

Concentration of corticosterone in plasma was estimated by the use of high performance liquid chromatography (HPLC) using betamethasone as an internal standard [35].

Cholinesterase activity determination

Cholinesterase activity in blood, plasma and erythrocytes, as well as in the brain was assayed with the modified Ellman's method using the Acetylcholinesterase Multi Reagent 96 Well Kit, Cat. No. 1418-500-K, IKZUS ENVIRONMENT, according to the manufacturer's protocol. (Please note that this method is not selective for AChE. Acetylcholinesterase is the only cholinesterase present in erythrocytes. However, in the rat and other rodents, two cholinesterases which can be targeted by an OP, AChE and butyrylcholinesterase (BuChE), both are present in plasma and in the brain [36]. Therefore, the use of the term: "cholinesterase – ChE", was preferred in the present work). Enzyme activity was determined be the use of the Multiskan Ascent Thermo Labsystems spectrophotometer for microplates and the data was recalculated using the equations specified in the manual.

Statistics

Differences were assessed using the Kruskall-Wallis test in Dunn's modification [37]. The trend analysis was performed be the use of Jonckheere's test [38]. The differences were regarded as significant when the probability of the null hypothesis was < 0.05.

RESULTS

A comparison of the CVP effect on the blood CORT concentration in MET-treated and MET-untreated rats is shown in Figure 1. In MET-untreated rats, administration of CVP resulted, 3 h after the pesticide administration, in a dose-related rise in plasma CORT concentration (Jonckheer's trend test, p_j < 0.005). In rats receiving
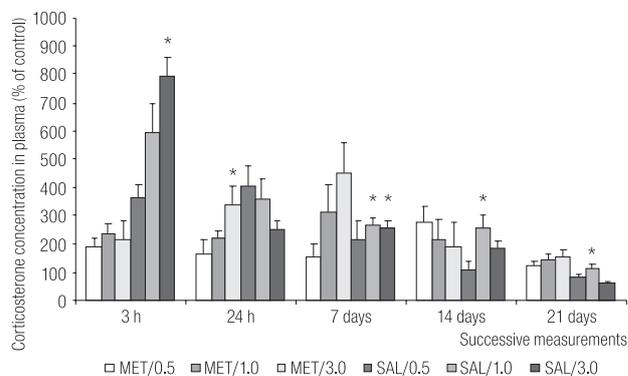
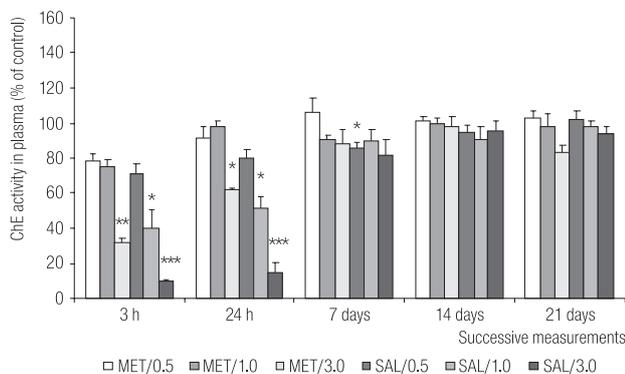* $p < 0.05$ compared to control.
Metyrapone at doses of 100 mg/kg *i.p.* was administered 5 times at 24 h intervals; the first metyrapone dose was given 2 h before the CVP administration. Data (means ±SEM, N = 4–5) are presented as percent of the values measured at the same time points in the respective control groups (SAL/0.0, or MET/0.0).
In the control groups the plasma corticosterone concentration ranged from 12.19 to 29.47 ng/ml.

Fig. 1. Diagrams illustrating changes in the plasma corticosterone concentration following CVP (0.5, 1.0 or 3.0 mg/kg *i.p.*) administration to metyrapone-treated and metyrapone-untreated rats

the highest (3.0 mg/kg) CVP dose, the rise was almost eight-fold as compared to the control (SAL/0.0) group. Significant increase in plasma CORT concentration was noted on day 7 (CVP dose 1.0 and 3.0 mg/kg) and day 14 (CVP dose 1.0 mg/kg). In the group which was given the highest CVP dose, the plasma CORT concentration on day 21 was significantly reduced compared to the control. In the MET-treated rats, CVP administration induced no clear-cut changes in plasma CORT concentration; a significant increase was noted solely in the MET/3.0 group and only at the 24 h time point.

Figures 2, 3 and 4 illustrate the dynamics of the CVP-induced changes in ChE activity in blood (plasma and erythrocytes) and brain of MET-treated and MET-untreated rats. In the MET-untreated rats the CVP administration resulted in a dose-dependent decrease of the ChE activity in all the compartments studied. This effect was most pronounced 3 and 24 h after the pesticide administration.

* p < 0.05, ** p < 0.01, and *** p < 0.001 compared to control.
Remaining description as in Figure 1.
The control values in the plasma ChE activities ranged
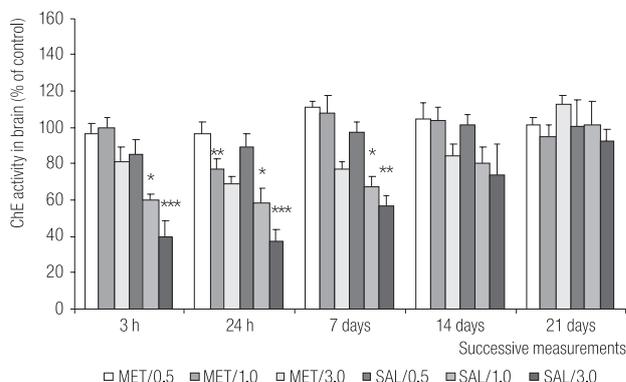1.02–1.89 μmol/min per 1 ml.

Fig. 2. Diagrams illustrating changes in the plasma ChE
activities following CVP (0.5, 1.0 or 3.0 mg/kg *i.p.*)
administration to metyrapone-treated and metyrapone-
untreated rats

Restitution of the enzyme activity was the fastest in plasma;
in all groups it was complete by day 7, whereas in the erythro-
cytes and the brain of rats given 1.0 or 3.0 mg/kg, at that time
the ChE activities were still significantly reduced at that time.
By day 21, restitution was complete in all compartments.
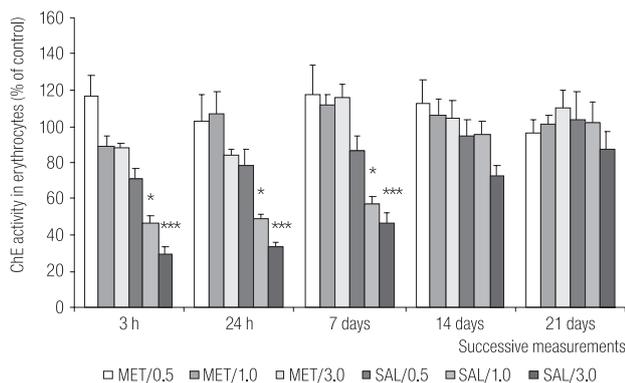
* p < 0.05 and *** p < 0.001 compared to control.
Remaining description as in Figure 1.
The control values in the erythrocyte ChE activities range
14.10–22.40 μmol/min per 1 ml.

Fig. 3. Diagrams illustrating changes in the erythrocyte ChE
activities following CVP (0.5, 1.0 or 3.0 mg/kg *i.p.*)
administration to metyrapone-treated and metyrapone-
untreated rats

* p < 0.05, ** p < 0.01 and *** p < 0.001 compared to control.
Remaining description as in Figure 1.
The control values in the brain ChE activities ranged
0.17–0.25 μmol/min per 1 mg protein.

Fig. 4. Diagrams illustrating changes in the brain ChE activity fol-
lowing CVP administration in metyrapone-treated and metyrapone-
untreated rats

In the MET-treated rats, CVP administration at the 0.5
or 1.0 mg/kg dose resulted in no significant alterations in
the ChE activity in any of the compartments tested. In rats
given CVP at the 3.0 mg/kg dose, the plasma ChE activity was
significantly reduced at 3 and 24 h, and that of the brain ChE
at 24 h after the pesticide administration. In both those com-
partments, however, the reduction was less pronounced than
that noted in the MET-untreated rats given the same CVP dose.

DISCUSSION AND CONCLUSIONS

As expected, the CVP administrations resulted in:
– a decrease in the ChE activities in blood and in the brain;
  in all studied compartments, the magnitude of this ef-
  fect was positively correlated with the CVP dose; the
  decrease was most pronounced in plasma but, generally,
  the differences between compartments were not great;
  the enzyme activity returned to normal within several
  days at a rate correlated with the dose (faster at lower
  doses) and the compartment (faster in case of plasma);
– an increase in the serum CORT concentration,
  i.e. the CORT response.

Also in this case the magnitude of this effect was positively correlated with the CVP dose. It disappeared within 24 h. (The intervals between successive measurements did not enable a more precise assessment of the duration of the CORT response in the present study).

Both effects reported above are characteristic for the majority of OPs, including CVP [13,31] and require no discussion. The most important issue is the effect of MET. The results show that the treatment resulted in reduction of the CVP-induced CORT response, markedly weaker ChE inhibition and accelerated recovery of the enzyme activity in all studied compartments. It is worth noting, however, that in the case of the plasma ChE the protective effect of the MET treatment was weaker, especially in the MET/3.0 group vs. remaining compartments. The most likely source of this difference may be the large contribution of BuChE to the plasma ChE activity; BuChE is known to be inhibited more effectively by most OPs than AChE [39].

MET is a known inhibitor of the CORT synthesis [32,40]. Therefore, the absence of the CORT response in the MET-treated rats of the present study was the expected outcome. A number of published works allowed one to predict that a blockade of the CORT synthesis might somehow modify the CVP effect: the magnitude of the ChE inhibition and/or the rate of the restitution of the enzyme activity. They do not allow, however, to predict the trend of the expected change. A transient rise in blood CORT concentration is a known response to various stressors. However, exposure to stressors results also in changes in ChE activities and an involvement of CORT in this effect seems quite likely. Kaufer et al. [10,11] for example, observed increased AChE synthesis in the neocortex and hippocampus after exposure to a physical stressor or an OP exposure. Increased AChE activity after exposure to stressors was also reported by other authors [18–21,41–44]. An involvement of CORT in these effects is suggested by some *in vitro* observations on PC 12 cells which showed an increase in AChE catalytical activity and AChE RNA

synthesis after incubation with CORT [28]. Based on the above observations, one might expect an amplification of the OP-induced ChE inhibition and/or slowing down the rate of restitution of the activity in the MET treated animals. An effect of this kind, i.e. "... decreased rate of regeneration of brain and striate muscle acetylcholinesterase..." was noted in adrenalectomized mice after sublethal poisoning with diisopropylphosphofluoridate (DFP) [29]. In the present study the effect of MET treatment, i.e. the compromised ChE inhibition and the accelerated restitution, was apparently opposite to that cited above, thus raising the question whether it could be due to a MET-induced inhibition of the CORT synthesis. Some authors reported a reduction in AChE activity after stress [22–24,44]. Similar effect was observed in striate muscles of animals treated with CORT [26,45] or dexamethasone, a syntetic glucocorticoid [27]. These observations suggest that an excess of glucocorticoids might downregulate, directly or indirectly, the ChE activity. If so, then in the case of the OP poisoning, the induced CORT response might somehow amplify the effect of the pesticide, i.e. the reduction in ChE activity. In such a case, the weaker reduction in ChE activity in MET-treated rats after CVP exposure could be explained as a result of the absence of the CORT response (i.e. the transient excess of glucocorticoids).

The above conjecture may be confirmed by administering CVP to adrenalectomized and CORT-supplemented rats. At present we are not in possession of our own data from an experiment like that. However, some speculations based on several published reports seem to be reasonable. It has been shown, for example, that adrenalectomized animals (rats) do not differ from controls (unoperated or sham operated) with regard to AChE inhibition after an OP exposure [15,25]. Moreover, it has been found that atropine pretreatment in soman-intoxicated rats prevented the CORT response but had no effect on the level of AChE inhibition [14,15]. The above observations suggest that in case of an OP intoxication, unlike in case of exposure to

nonchemical stressors, there is no relationship between the CORT response and the AChE activity. If so, the MET effects observed in the present experiment do not seem to be attributable to inhibition of the CORT synthesis.

A search through the literature reveals that results similar to these obtained in the present study, i.e. a decrease of the inhibitory effect of OP on ChE activity, were achieved with the use (as prophylaxis or therapy) of memantine [46–48], N-acetylcysteine (NAC) [49–51], or caffeic acid [52]. NAC and caffeic acids are known antioxidants. The biochemical alterations produced by memantine (a noncompetitive inhibitor of the NMDA receptors) also suggest a protective action against the generation of oxidative stress, i.e. over-production of reactive oxygen or nitrogen species in the brain and peripheral tissues [48].

Oxidative stress is regarded as one of the main adverse effects of OP intoxication [53]. According to some authors, oxidative stress is responsible for the long-term degenerative alterations in the nervous system which may follow an OP exposure [48,54]. Ample evidence shows that in condition of oxidative stress the activity of cholinesterases is inhibited and that the excess of hydrogen peroxide is the main culprit [55–58]. It follows from the above that two factors determine the reduction in cholinesterase activities in OP intoxication: first is the inactivation due to the OP-ChE binding and the second, which results from the former, the oxidative stress. This explains why treatment with antioxidants may result in reduction of the ChE inhibition induced by an OP exposure. It seems quite probable that in the present experiment we were dealing with a similar process, that is a reduction of the OP-induced oxidative stress. It is generally recognised that MET acts not only to inhibit 11β-hydroxylase, but also other enzymes of the cytochrome P450 system [32,59]. This system plays a key role in the metabolism of numerous xenobiotics. Its activity, however, is the main source of reactive oxygen species in at least some mammalian tissues [60,61]. Thus, inhibition of the P450 system may prevent generation of oxidative stress.

Exposure to CVP, like exposure to other OP insecticides, results in generation of reactive oxygen metabolites and oxidative stress [62,63]. It is also known that cytochrome P450 participates in the CVP metabolism [64,65] and it is likely that the P450 activity is an important cause of the CVP-induced oxidative stress. Thus, inhibition of the P450 system by MET limits the oxidative stress generation and, owing to that, protects, albeit partially, ChE from inhibition. However, on the other hand, inhibition of the P450 system may result in slowing down the CVP metabolism, what should increase rather than decrease its toxicity. Keeping this in mind, the MET effect observed in the present work might suggest that the benefits from the reduction of the oxidative stress, assessed on the basis of the reduction in ChE activity, are greater than the losses resulting from slowing down the CVP metabolism.

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