

## EFFECTS OF STRESS PRETREATMENT ON THE DYNAMICS OF BLOOD CHOLINESTERASE ACTIVITY AFTER EXPOSURE TO AN ORGANOPHOSPHORUS PESTICIDE IN THE RAT

Sławomir Gralewicz, Radosław Świercz, Piotr Lutz, Dorota Wiaderna, Wojciech Wąsowicz

Department of Toxicology and Carcinogenesis, Nofer Institute of Occupational Medicine, Łódź, Poland

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**Abstract:** A single i.p administration of 1.0 mg/kg of chlorphenvinphos (CVP), an organophosphorus pesticide, results in an acute stress response, evidenced by a marked (6–7 fold) rise in plasma corticosterone (CORT) concentration, and a diminished behavioural sensitivity to amphetamine (AMPH) three weeks postexposure. Surprisingly, in rats subjected to a single series of inescapable electric footshocks (60 10 msec triplets of 3.0 mA, 2 msec, square pulses during 20 min – IF ) two weeks prior to the CVP exposure, these effects are not observed. It has been assumed that the reduced effectiveness of CVP might be related to some persisting alterations in the functional state of the cholinergic system. The aim of the present work was to discover whether and in what way the IF pretreatment affects i) the cholinesterase activity in blood, and ii) the dynamics of the alterations in the cholinesterase (ChE) activity following the CVP exposure. The experiments were performed on 3 mo. old, male Wistar rats. In the first experiment, the blood samples were taken from the tail vein 15, 60 and 180 min after the IF. In the second experiment, the rats were pretreated with IF and 14 days later given 1.0 mg/kg of CVP i.p. Blood samples were taken 15 min, 60 min, 180 min, 24 h, 7 days, and 14 days after the CVP exposure. In the first experiment no differences in the ChE activity in plasma (pChE) and erythrocytes (rbcChE) were found between the shocked and control rats. In the second experiment, however, in rats pretreated with IF the rbcChE activity of was reduced by CVP less and pChE activity returned to normal faster than in rats not pretreated with IF. The results confirm that exposure to IF, a nonchemical stressor, induces some long-lasting adaptive changes which render the cholinergic system less susceptible to the harmful action of ChE inhibitors. It has been hypothesized that the changes consist in an increase of the antioxidant potential in blood and possibly other tissues.

**Address for correspondence:** Sławomir Gralewicz, Department of Toxicology and Carcinogenesis, Nofer Institute of Occupational Medicine, Teresy 8, 90-950 Łódź, Poland. E-mail: [gralslaw@imp.lodz.pl](mailto:gralslaw@imp.lodz.pl)

**Key words:** cholinesterase inhibitor, cholinesterase, corticosterone, amphetamine, stress, behaviour, rat.

### INTRODUCTION

Stress may result in profound and prolonged health consequences [23, 47]. In everyday life exposure to variable stressors, i.e. factors able to activate the hypothalamo-pituitary-adrenal (HPA) axis, is inevitable. Stressors are also numerous chemicals commonly used in industry, agriculture and household which makes it likely that at least some of the health consequences of overexposure to such

substances are stress-related. It appears, from laboratory data, however, that the subject's vulnerability to a chemical stressor may be altered – increased or reduced – by stressogenic experiences occurring days or weeks before the exposure. An example of the increased vulnerability is the augmented behavioural sensitivity to psychostimulants, which may develop after exposure to various chemical and nonchemical stressors [3, 38, 44]. Findings of a decreased vulnerability are less frequent. A reduced response to a



cholinergic agonist was reported after a social stress [7, 13]. Similar results, although obtained with the use of different stressors and in different experimental settings, were also reported by other authors [27].

Evidence suggesting decreased sensitivity to chemical stressors following pretreatment with a nonchemical stressor was also obtained in our laboratory [15]. The chemical stressor used in our studies was chlorphenvinphos (2-chloro-1(2, 4-dichlorophenyl) vinyl diethyl phosphate – CVP), an organophosphate pesticide. The nonchemical stressor was a series (three/min, for 20 min) of painful inescapable electrical footshocks (IF) applied to the animal paws through metal floor. In “naive” rats i.p. administration of 1.0 mg/kg of CVP results in a 6–7 fold increase of the plasma corticosterone (CORT) concentration lasting several hours. Three weeks later, the rats show a reduced behavioural response to an amphetamine (AMPH) challenge indicating that some long-lasting alterations, possibly concerning the brain cholinergic-dopaminergic balance, have developed [14]. Surprisingly, neither the increased CORT response, nor the hyposensitivity to AMPH, was found in rats pretreated with IF two weeks prior the CVP exposure [15].

We have found recently that in rats treated with metyrapone, a blocker of the CORT synthesis, before the CVP exposure the response to AMPH is not altered (manuscript in preparation). This suggests that this effect (i.e. the reduced sensitivity to AMPH) is probably related somehow to the CVP induced CORT response. Thus, its absence in the IF pretreated rats is understandable. The question remains, however, why in the IF pretreated rats the CORT response to CVP was reduced.

The main stimulus responsible for the increase in the HPA axis activity after the CVP exposure is the hyperactivity of the cholinergic system resulting from inhibition of acetylcholinesterase (AChE). It is known that exposure to stressors can induce alterations in expression and activity of cholinesterases in the central nervous system and in the periphery. It has been found, for example, that exposure to physical or chemical stressors stimulate the AChE synthesis, which results in an increased concentration of this enzyme for some time after the stressful experience [24, 25]. Thus, it is quite likely that the suppression of the CORT response to CVP in our experiments was due to an IF induced increase in ChE activity and, owing to that, diminished effectiveness of the CVP dose.

The purpose of the present experiments was twofold. First, to find the changes in ChE activity following the IF treatment. Second, to compare the dynamic of the alteration in the ChE activity after CVP exposure in nonstressed rats and rats pretreated with IF. The present experiment concerns changes which may be detected in blood, i.e. in red blood cells (rbc) and plasma (p). Two arguments justified this choice. First, it has been shown that in the organophosphate poisoning the changes in rbc ChE activity correlate well with changes in the brain ChE [39]. Second,

unlike samples of brain tissue, blood samples may be taken repeatedly from the same animal at different time points.

## MATERIALS AND METHODS

**Animals.** The experiments were performed on adult (3–4 months old), Wistar rats, males, outbreds, obtained from our Institute’s breeding facility. The animals were acclimatised for two weeks before the start of the experiments. They were housed singly in rat cages. The temperature (22°C), humidity (50–60%) and the light/dark cycle (12/12 h with light on from 0600 to 1800 h) 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. All animal use procedures were approved by the local Bioethical Committee (Decision No 43 ŁBS72/2007).

**The FS equipment and procedure.** The apparatus for the IF application and the shocking procedure were identical as described earlier [15]. Rats of the shocked groups received 60 footshocks (three shocks/min during 20 min) in a shock cage equipped with a metal grid floor which could be electrified. Each shock consisted of a 10 msec triplet of 3.0 mA, 2 msec, square pulses. Immediately after the last shock, the rat was returned to its home cage. Control animals were placed in the shock cage for 20 min but they were not shocked. Each time before testing the next rat, the droppings collector and the floor of the cage were cleaned with a wet cloth.

**Chlorphenvinphos.** Chlorphenvinphos [2-chloro-1(two, 4-dichlorophenyl) vinyl diethyl phosphate], technical grade, was obtained from the manufacturer (ORGANIKA-AZOT, Jaworzno, Poland). It was diluted with olive oil (OIL) and administered intraperitoneally at a single dose of 1.0 mg/kg. b.w. (ca 1/10 DL50). The volume of the injected solution was 1.0 ml/kg b.w. The CVP dose and the administration procedure were identical as employed in the earlier study [15]. In the rat the behavioural symptoms produced by i.p. administration of 1.0 mg/kg CVP are inconspicuous (motor slowing), the maximum inhibition of acetylcholinesterase activity in blood and in the brain is about 50% on average, and the enzyme activities normalize within 7–14 days [54].

**Determination of ChE activity in blood.** Blood samples were collected from the rat tail with the “nick” method at predetermined time points to heparinized vials. The sample volume was about 200 µl. The ChE activity was assayed with the modified Ellman’s method using the Acetylcholinesterase Multi reagent kit, Cat. No. 1418-500-K, IKZUS ENVIRONMENT, and observing the manufacturer’s protocol.



**Table 1.** Groups and procedures in Part 2 of the experiment.

Group	Procedure	
	Shock cage experience	Type of chemical exposure
C 1 (n=6)	None	oil
C 2 (n=6)	Placed in the shock cage but not shocked	oil
S (n=6)	Placed in the shock cage and shocked	none
S-O1 (n=6)	Placed in the shock cage and shocked	oil
S-CVP (n=6)	Placed in the shock cage and shocked	CVP
CVP (n=6)	Placed in the shock cage but not shocked	CVP

**Procedure.** The experiment was performed in two parts. In Part 1 the acute effect of the IF on the blood ChE activity was studied. In Part 2 the effect of the IF pretreatment on the magnitude and dynamics of the ChE inhibition resulting from an acute exposure to CVP was tested.

**Part 1. Groups and procedure.** Part 1 of the experiment was performed on three groups of rats ( $n = 6$  in each group): two Control groups (C 1 and C 2) and the Stress group (S). The C 1 group were naive animals (they were never placed in the shock cage). Rats of the C 2 group were placed in the shock cage for 20 min but received no footshocks. Rats of the S group were shocked in the shock cage in the way described above. In the S group and the C 2 group the blood samples were collected 15, 60 and 180 min after the stay in the shock cage. In the C 1 group the samples were collected three times; the second sample taken 45 min and the third 165 min after the first sample.

**Part 2. Groups and procedures.** Six groups of rats, ( $n = 6$  in each group), were used in Part 2 of the experiment. There were two control groups (C 1 and C 2), the Stress group (S), the Stress-Oil group (S-O), the Stress-CVP group (S-CVP), and the CVP group (CVP). The groups and the procedures are shown in Table 1.

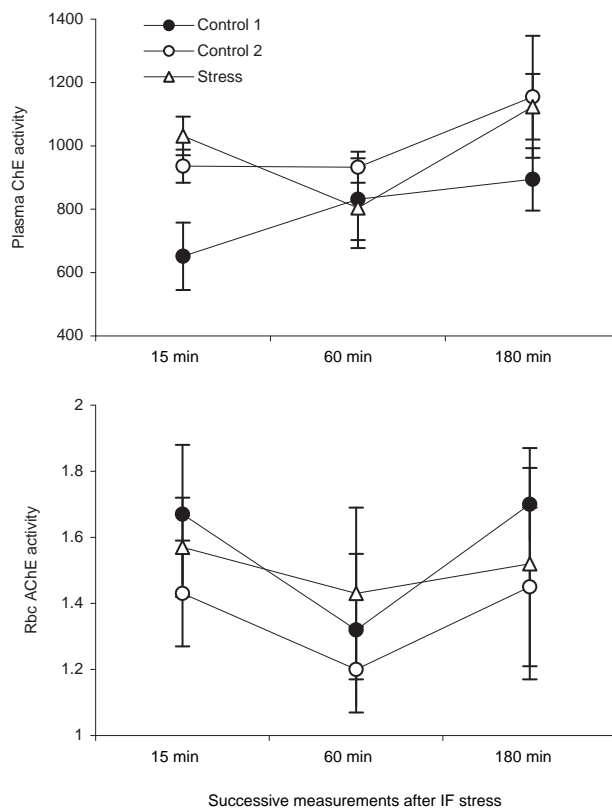
In the S group, blood samples were taken three times on day 15 at intervals of 45 and 120 min, and then on days 16, 22, and 29 after the IF. In the remaining groups, blood samples were taken 15 min, 60 min, 180 min, 24 h, 7 days and 14 days after the i.p. administration of oil or CVP.

**Statistics.** A two-factor parametric ANOVA for repeated measurements was used for statistical comparisons. When the group  $\times$  measurements interaction was significant the Tukey test was used for pairwise between groups comparisons within successive measurements.

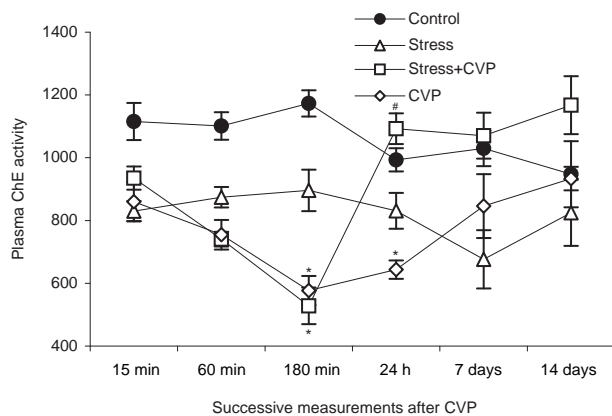
## RESULTS

**Part 1. Effect of footshock stress on blood cholinesterase activity.** The groups did not differ significantly in body weight on the day of shocking (data not shown). Results illustrating the effect of the IF on ChE activity in plasma or red blood cells are presented in Figure 1. Neither in the case of pChE nor rbcChE the effects of the group factor, the measurement factor or the group  $\times$  measurement interaction were significant.

**Part 2. Effect of stress pretreatment on the CVP induced alterations in ChE activities.** There were no significant differences between groups in body weight throughout the experiment (data not shown). A preliminary comparisons of the data concerning both, pChE and rbcChE, showed no differences between the C 1 and C 2 group, as well as between the S and S-O group, indicating that the effects of the 20 min visit to the shock cage (group C2) or the ip.injection of unadulterated oil (group S-O) could be ignored. Therefore, control groups were pooled into one control group (C,  $n=12$ ) and the Stress and Stress-Oil groups into one Stress group (S,  $n=12$ ). Thus, the comparisons were made for four groups: C, S, and S-CVP and CVP group. Results concerning the pChE activity are pre-



**Figure 1.** pChE (upper diagram) and rbcChE (lower diagram) activities in rats after a 20 min series of inescapable electric footshocks (IF). The pChE activity is expressed in  $\text{nM}/\text{min} \times 1 \text{ ml}^{-1}$ . The rbcChE activity is expressed in  $\text{nM}/\text{min} \times 1 \text{ mg Hb}^{-1}$ . Each point represents group mean. Error bars represent standard errors of the means (SEM).



Groups denotation: Control – rats not pretreated with FS, Stress – rats pretreated with FS but not injected with CVP, Stress+CVP – rats pretreated with FS and given CVP, CVP – rats given CVP but not pretreated with FS. \*  $p < 0.05$  compared to Control, #  $p < 0.05$  compared to CVP.

**Figure 2.** Diagrams illustrating the effect of FS pretreatment on the CVP (1.0 mg/kg, i.p.)-induced changes in the pChE activity in rats. The pChE activity is expressed in  $\text{nM}/\text{min} \times 1 \text{ ml}^{-1}$ . Each point represents group mean. Error bars represent standard errors of the means (SEM). FS – a 20 min series of inescapable footshocks given 14 days before the CVP exposure.

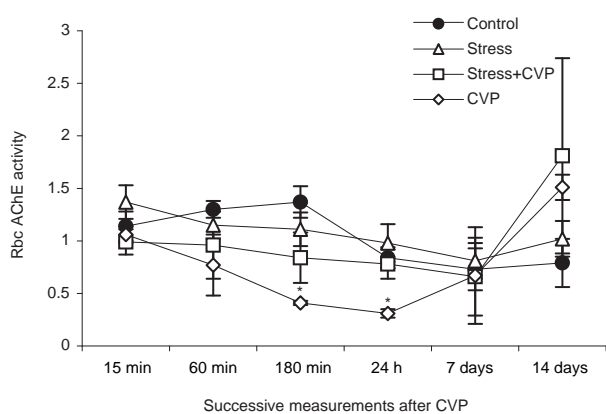
sented on Figure 2. The ANOVA revealed that the group  $\times$  measurements interaction was significant ( $F(3,32) = 40,18$ ,  $p < 0.0001$ ). Detailed comparisons showed no significant difference between the C group and the exposed groups (S-CVP and CVP) in the first time point (15 min postexposure). In the CVP and the CVP-S group 180 min after the injection, the pChE activity was significantly lower than in the control group. However, in the S-CVP group, unlike in the CVP one, the restitution of the pChE activity was unexpectedly fast: 24 h after the treatment the pChE activity in this group approached that in the C group, whereas in the CVP group it was still significantly decreased compared to the C group as well the S-CVP-S group.

Regarding the rbcChE, significant differences between the successive measurements were found only in the CVP group; at the third and the fourth time points, i.e. 180 min and 24 h after the CVP exposure, the rbcChE activity was significantly lower than in the first and the last time points i.e. 15 min and 14 days after the CVP exposure. The third and the fourth time points were the only ones where significant differences between groups were found; at these points, the rbcChE activity in the CVP group was significantly lower than in the control group. In case of the Stress+CVP group, the difference was not significant (Fig. 3).

## DISCUSSION

The results of Part 1 indicate that up to three hours after the FS, the ChE activity in blood, plasma and erythrocytes, was within the normal range.

Alterations in ChE (AChE) activity after exposure to various stressors have been described in a number of reports, most of which concern the brain. Some authors reported an increase [24, 25, 35, 45], in some but a decrease



Groups denotation: Control – rats not pretreated with FS, Stress – rats pretreated with FS but not injected with CVP, Stress+CVP – rats pretreated with FS and given CVP, CVP – rats given CVP but not pretreated with FS. \*  $p < 0.05$  compared to Control.

**Figure 3.** Diagrams illustrating the effect of FS pretreatment on the CVP (1.0 mg/kg, i.p.)-induced changes in the rbcChE activity in rats. The rbcChE activity is expressed in  $\text{nM}/\text{min} \times 1 \text{ mg Hb}^{-1}$ .

in other brain regions [11], or only a decrease [9, 10, 19]. Generally, findings reporting a decrease in ChE activity following stress are more common. There are a number of reports from studies aimed at finding out whether and how exposure to various stressors influences the toxicity of pyridostigmine, a carbamate used in the prophylaxis of poisoning with nervous gases [1, 5, 6, 20, 26]. In none of these studies was the brain ChE activity altered after exposure to physical or psychological stressors alone.

Unlike in the case of the brain, there are only a few reports dealing with the effects of stress on blood ChE. Reports concerning the effects of unavoidable footshocks are lacking, and results obtained with the use of other stressors vary. For example, some authors have reported that acute as well as chronic exposure to a physical (strenuous exercise) or chemical (ethanol) stressor, or a combination of both, results in an increased butyrylcholinesterase (BUChE) activity in plasma [18, 19, 20]. Other authors, using restraint, reported opposite effect [51]. Human data concerning the rbcChE suggest an increase in activity following intense muscular effort [40, 41, 50], or psychical stress (anxiety related to imminent surgery) [52]. In rats, a decrease in rbcChE activity was observed following exposure to an unspecified stressor [30]. On the contrary, Bairredy et al [5] found no changes in the ChE activity in full blood and the diaphragm muscle following stress. As it appears from the above, the pattern of the stress induced changes in the ChE activity in blood is not uniform, which is probably due to the variability in the type of stressors and the stress regimes as well the experimental material (strain and/or species) used. In this context, the negative results of Part 1 of the present study (i.e. the absence of overt changes in blood ChE activity shortly after stress) are not surprising. In case of the results of Part 2 the situation

is different. They indicate that in the long term the shocking exerted some effects on the blood ChE activity, namely it resulted in: i) a reduced susceptibility of the rbcChE to inhibition by CVP, and ii) an accelerated restitution of the pChE activity following CVP exposure. There is no doubt that reduced ChE activity and cholinergic hyperactivity are the main factors responsible for the CORT response to CVP. Therefore, the results of Part 2 of the present experiment could account for the reduced CORT response to CVP as well as the absence of the long-lasting effect: the hyposensitivity to AMPH in the FS pretreated rats [15].

We found no reports from studies dealing with the effects of stress pretreatment on the organophosphate-induced changes in ChE activity in blood. With regards to the brain, it has been reported recently that long term stressing with a combination of various stressors does not influence the AChE inhibition by chlorpyrifos [16]. However, the experimental design and the stressors used in that study differed markedly from those employed in the present experiment.

The basic question emerging from the results of the present study is: what is the nature of the FS induced change responsible for the reduced cholinotoxicity of CVP? An answer to this question is suggested by data that make it likely that binding organophosphate to the cholinesterase molecule is not the only factor responsible for the enzyme inactivation in organophosphate poisoning. Another is the oxidative stress. It has been shown that in conditions of oxidative stress the activity of cholinesterases is reduced [29]. The main factor responsible for this reduction is an overproduction of H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide) which "... oxidizes susceptible amino acid residues, such as methionine, tryptophan, cysteine and selenocysteine, in the structure of protein and peptides which in turn can severely affect the function..." [48, 49]. There are a number of reports documenting the ability of organophosphates (and generally cholinesterase inhibitors) to generate oxidative stress in the central nervous system and in the periphery [2, 12, 32, 53, 62]. CVP is no exception which has been confirmed recently [28]. It has also been shown that in conditions of oxidative stress cholinesterases are inhibited regardless of the nature of the stress-inducing agent [57, 58]. Apart from the classical cholinesterase inhibitors: organophosphates and carbamates, a decrease in cholinesterase activity was observed to result from exposure to pyrethroids [22, 63], polychlorinated biphenyls [33, 59, 60], arginine [61], or after a strenuous muscular effort [40, 50]. In all cases cited above, antioxidants (vitamin C and/or E) given as prophylactics or a cure, prevented or ameliorated the effect on cholinesterase activity confirming the role of the reactive oxygen species (ROS) as the causative factor.

The observations cited above provide strong support for the assumption that in organophosphate poisoning oxidative stress is an important factor contributing to the decrease in cholinesterase activity (this concerns the brain as well as the periphery, including blood). It follows from the

above that any factor able to increase the antioxidant potential of the organism may reduce its vulnerability to organophosphates. (In fact, it has been found that in fish the effects of organophosphate poisoning, including the decrease in ChE activity, may be totally prevented or ameliorated by pretreatment or treatment with antioxidants [42, 43]. It has also been shown that intravenous administration of antioxidants after exposure to organophosphate pesticides prevents ROS formation in erythrocytes [56] and accelerates restitution of cholinesterase activity in plasma [21].

Thus, the reduced effectiveness of CVP in the FS pretreated rats in Part 2 of the present study (i.e. the insignificant rbcChE inhibition and accelerated plasma ChE restitution) could be understandable if the antioxidant potential of these animals was augmented. The question is: whether such an effect could be induced by the FS pretreatment? It has been shown that exposure to various stressors, including foot-shock [37], restraint [4, 8, 36, 66], immobilisation [34, 46], and social isolation [17], ethanol ingestion [55], induce oxidative stress in the brain and peripheral organs. The main factors responsible for these effects are stress hormones, mainly glucocorticoids [64, 65]. In the case of strong and long-lasting stressors, the resulting alterations in cell structure and functions may be deleterious which is evidenced by the known effects of an excess of glucocorticoids in the central nervous system [23, 47]. On the contrary, weak and short-lasting stressors may result in changes increasing the organism's resistance (ability to cope) to the same as well to other stressors. The changes include an increase in the antioxidant potential (increased superoxide dismutase, catalase and glutathione peroxidase activities and glutathione content) [31]. Based on the above, the supposition that an augmentation of the antioxidant potential in blood (and possibly in other tissues) was the main factor responsible for blunting the anticholinesterase effect of CVP in the FS pretreated rats is a likely one. Its reliability will be checked in future experiments.

## CONCLUSIONS

1. In the rat exposure to a series of unavoidable shocks exerts no immediate effect on the cholinesterase activity in blood, but it blunts the anticholinesterase effect of an organophosphate pesticide given two weeks postexposure.
2. It has been concluded that exposure to a physical stressor results in adaptive changes which are manifested in reduced sensitivity to organophosphate pesticides. Literature data suggests that these changes may consist in an increased antioxidant potential in blood and possibly other tissues.

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