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THE ROLE OF SOME ENDOGENOUS AND EXOGENOUS NITRIC OXIDE DONORS AS A POSSIBLE ANTIDOTE AT POISONING FROM CARBAMATE PESTICIDES

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Abstract: Enzyme mapping of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in certain segments of the brain of rabbit and rat was done in this work. The differences in enzyme activity, characterized histologically was attributed to the BChE, an enzyme, which plays a major role in the defense mechanisms of the body and mainly the nervous system against poisons that damage cholinergic functions.

It was first found that certain nitric oxide donors, such as L-Arginine and Na-Nitroprusside stimulate the activity of both esterases – AChE and BChE. This stimulatory effect of L-Arginine (respectively of nitric oxide (NO) on the AChE activity ranges from several percents to 1-2 fold compared to the controls. The activation of BChE activity reaches an increase of 3-8 folds. This stimulation of enzyme activity was attributed to the influence of NO. The effects of Na-Nitroprusside are biphasic – activation at very low concentrations (0.05-0.1 mM) and progressive concentration-dependent inhibition with increasing concentrations of the reagent. This inhibitory effect is due to specific metabolic products released during decomposition of Na-Nitroprusside.

The stimulating effect of NO donors L-Arginine and Na-Nitroprusside on the studied enzyme activity were considered significant. Therefore, they can be used as an effective antidote to poisoning by organophosphate and carbamate pesticides, and intoxication by chemical warfare agents.

INTRODUCTION

Cholinergic system plays a central role in neurotransmission in animals and humans. The esterases are important components of this system, that control the overall health status, behavior and the manifestation of some serious diseases (Ivanov, 2006).

A prerequisite for our consideration on this topic were data from a monitoring survey of the influence of some donors of NO on bioenergetic and synaptic processes and mechanisms of regulation.

Cholinesterases in the body are of two types - ***Acetylcholinesterase*** also known as erythrocyte cholinesterase (AChE; CE 3.1.1.7) and ***Pseudocholinesterase*** also known as *plasma cholinesterase* and *butyrylcholinesterase*, found primarily in the liver. (BChE; EC 3.1.1.8) (or BuChE).

AChE is an enzyme that hydrolyzes acetylcholine to choline and acetic acid. It is mainly found at neuromuscular junctions and cholinergic synapses in the central nervous system. This enzyme has a very high catalytic activity — each molecule of AChE degrades about 25 000 molecules of acetylcholine per second. The choline produced by the action of AChE is recycled through reuptake, back into nerve terminals where it is used for synthesis of new acetylcholine molecules.

Anticholinesterase agents inhibit the enzyme activity. All compounds that inhibit cholinesterase are potent neurotoxins, pesticides, insecticides, medicines, chemical warfare agents, etc. (Henk et al., 2012; Cowel, 2013).

Butyrylcholinestrase is much less studied in terms of its role as regulator and the consequentmechanism of physiological and mental functions and adaptation of organisms to specific disadvantages. Suggested references are a very small part of those that reveal the consequences of a genetic deficiency of this enzyme (Barta et al., 2001; Hidaka et al., 1997), as a factor for the elimination of drugs like heroin, cocaine, etc. (Browne et al., 1998, Zheng et al., 2008) and as a marker enzyme (Pohanka, 2013).

There are no published evidence on the effect of NO on AChE- and BChE activity.

The aim of the study was to monitor the role of nitric oxide (NO) through precursors of this neurotransmitter (L-arginine, Na-nitroprusside, nitroglycerin, amyl nitrate, etc.) on the activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in the brain and body of various species to seek pharmacological, physiological and therapeutic approaches to protect against poisoning with anticholinesterase pesticides, insecticides and chemical warfare agents.

MATERIALS AND METHODS

Preparation of enzyme fractions. Isolation of membrane and mitochondrial brain fractions was performed by the method of differential centrifugation. After preparation of the tissues from laboratory animals, they were placed in an ice bath, washed with extraction solution, dried on filter paper and the necessary structures were weighted. The tissue samples were cut with scissors on ice and homogenized in extraction solution (100 mM KCl, 1 mM $MgCl_2$, 0,1 mM EDTA-Na salt, 50 mM Tris-HCl (pH = 7,6) in the following ratio per gram tissue 1:9 (10 folds) or 1:4 (5 folds) with different homogenizers, according to the objectives of the study – metal blender, glass-glass, glass-teflon etc. Homogenization was performed twice for 60 seconds.

The resulting homogenate was filtered through gauze to separate foaming and residue fractions.

The homogenate was centrifuged at 3000 rpm (1500 g) for 25-30 min. The resultant pellet contains cell nuclei and damaged tissues, cells and undegraded particles. The supernatant contains the mitochondrial membrane fraction, used in this study. In other studies, the supernatant was centrifuged at 15 000 rpm (14 000 g) for 30 min. The resulting pellet contains the gross mitochondrial fraction and the supernatant contains impure membrane.

Laboratory animals. Male and female, white Wistar rats (200-250 g) and rabbits were used in the study. The investigation conformed to the international and national rules and regulation requirements for ethical attitude towards the animals.

Determination of acetylcholinesterase and butyrylcholinesterase activity.

The difference between the two types of cholinesterases is appointed to their respective preferences for substrates: acetylcholine is a substrate for AChE and butyrylcholine for BChE. We determined AChE and BChE activities by the method of *Elman et al.* (1961).

Esterase activities were measured by artificial substrate acetylthiocholine iodide (ATChI) or butyrylthiocholine iodide (BTChI). AchE or BChE release thiocholine (TCh) from ATChI or BTChI. The SH-groups of TCh reacts with dithionitrobensoic acid (DTNB), which is reduced to thionitrobensoic acid (mercaptonitrobensoic acid; MNB) – a yellow coloured anion with absorption maximum at λ=412 nm.

The intensity of coloration is proportional to the concentration of TCh.

Reactions were stopped by the addition of the specific blocker of AChE or BChE activity – eserine.

Enzyme activity was determined in samples containing 3,0 ml 50 mM K, Na-phosphate buffer (pH=7,6); 0,1 or 0,2 ml of the enzyme fraction (20-100 µg protein/sample) - 0,02 ml ATChI (21,67 mg/ml) or 0,02 ml BTChI (4,80 mg/ml); 0,2 ml of the corresponding reagent, followed by incubation for 30 minutes at

37°C. The enzymatic reaction was stopped with the addition of 0.1 ml 0,1 mM eserine salicilate. Finally, 0.1 ml DTNB was added and after 5 min the absorption at 412 nm was measured.

Control samples contained the same components as the experimental, but without substrate.

Determination of protein concentration. The protein concentration in the samples was determined by the method of Lowry,1961. Absorption of the samples was measured at λ=750 nm.

Statistic analysis. The significance of differences between control and experimental samples was estimated by Student`s t-test (Walpole at al., 2002).

RESULTS AND DISCUSSION

1.Enzyme mapping (screening) of cholinesterase activity in different areas of the brain of white rat and rabbit

Activities of AChE and BChE of membrane-mitochondrial fractions of different areas of rabbit and rat brains – (Fig. 1) were determined. This enzyme mapping allows to study the histological positioning of the individual components of this system - cholinergic, choline receptive or both and to compare the activities of both enzymes and evaluate their biological significance.

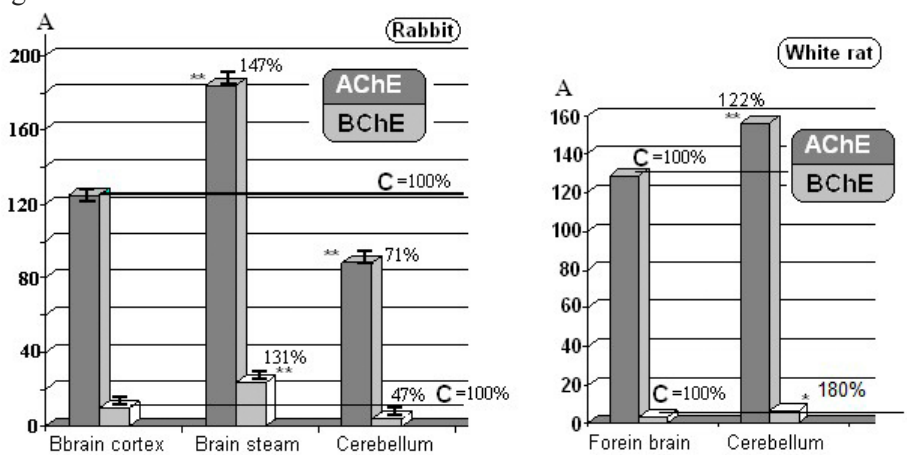

Figure 1. Activity of AChE and BChE in various fractions of brain of rabbits and rats.

A (enzyme activity) – μg hydrolyzed acetylcholine/mg protein/min; n=8-12; ±m (AChE) = 5-15 and ±m (BChE) = 0,65-0,90; significal differences: *-p<0,05, **-p< 0,001; this indications are valid for all figures and tables below. The precise values of the enzyme activity in some figures and tables are shown similarly in tabular form as % relative to control (100%).

The precise values of the enzyme activity to some of the figures are shown in tabular form and in percent (%) relative to controls (C=100%).

Tabular form data (see Fig. 1)

(*Rabbit brain*)			(*White rat*)	
Brain cortex	Brain steam	Cerebellum	Forebrain	Cerebellum
124,88	183,77	88,13 A (AChE)	28,30	156,09 A; AChE
100%	147%	71%	100%	122%
10,11	23,43	4,51 A (BChE)	3,67	6,60 A; BChE
100%	131%	47%	100%	180%

Different levels of AchE and BChE activity in different brain areas were established, which corresponds to the histochemical localization of different esterases in the brain. The activity of BChE in comparison to AchE in the brain is about 9%-10%. There is a proportional correlation between the activities of the two enzymes. This enzyme is an important biochemical marker in the brain as well as other organs and tissues (Pohanka, 2013). BChE plays an essential role in the mechanisms of normal physiological functions in the body's adaptation and prevention of poisoning by organophosphate and carbamate pesticide and abuse with narcotic agents (cocaine, heroin) and other toxic substances (Zheng et al., 2008).

In literature, there is ample evidence for preventive functions of BChE as a system that blocks various neurotoxic agents in plasma, haemolymph and tissues. This enzyme activity limits their harmful effects on the nervous system.

2. Influence of L -arginine on the activity of AChE and BChE in the mitochondrial-membrane fraction in different areas of the brain of rats and rabbits.

The influence of the nitric oxide donor L-Arginine on the activity of ACHE and BChE in various brain areas in a white rat is reported here.

Results show that L-Arginine in a wide concentration range (10-50 mM) stimulates AchE and BChE activity (Fig. 2). The pattern of increase of the enzymatic activity of both enzymes is similar for the two parts of the brain (forebrain and cerebellum). However, there are significant differences, related to the threshold concentrations of the beginning of the activation effect, and to the level of stimulation.

According to the experimental data in rat, AChE activity in the cerebellum is 65% higher than that in the forebrain. The proportion of activity of BChE is similar (55%).

Significant differences occur in the threshold level of activation, in which the foreign brain in terms of AChE is about 5 mM and for the cerebellum - at 25-30 mM. A similar dependence on the activating effect of L-Arginine on BChE activity in the forebrain and cerebellum was established.

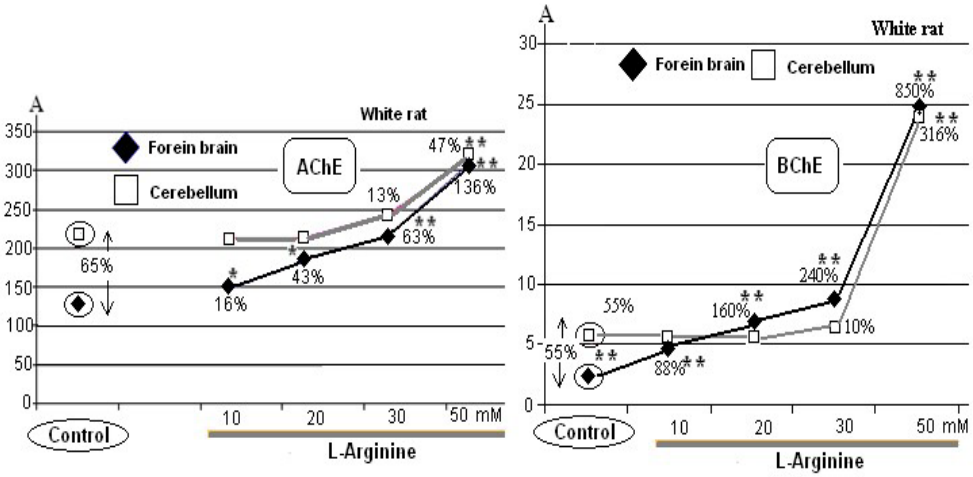

Figure 2. Influence of L-Arginine (10, 20, 30 and 50 mM) on the activity of AChE and BChE in membrane-mitochondrial fraction of forebrain and cerebellum of white rat. Other indications – see Fig. 1.

Activating efficiency of L-Arginine on forebrain AchE and BChE was much higher than that of the cerebellum, respectively, 136% and 47%. Remarkably high is the level of stimulation of BChE activity, which at 50 mM reached 850% for forebrain and 316% for the cerebellum.

The activation threshold concentration of L-Arginine on the activity of both enzymes is much lower for the forebrain (about 10 mM) than that for the cerebellum (25-30 mM).

The summary conclusion from the data in this series is that L-Arginine as the source of NO in a wide concentration range significantly activated both AChE and BChE activity in fractions from different brain areas. These effects are explained by the physiological and biochemical characteristics of these brain structures. The incentive effect on BChE activity is much higher (for example, 8.5 folds higher and about 3 folds higher respectively in forebrain and cerebellum), while activation on AChE activity to the forebrain and cerebellum is in the order of 136% and 47% respectively.

There are significant differences in the threshold concentration of the activating effect of the reagent. This explains the differences in the structural and functional components in the brain field.

This is the basis for the expansion of the research program related to BChE, which is the first link in the protection of organisms (including human who is under the influence of intoxication by various pesticides and chemical warfare poisonous substances). We must not forget the use of organophosphatus neurotoxic substances during World War II, in the Tokyo subway, in Iran and Syria in the recent past.

3. The influence of Na-Nitroprusside on activities of AChE and BChE in the mitochondrial-membrane fraction of different areas of the brain of rat and rabbit

Na-nitroprusside is a pharmacological substance (Na_2 [Fe $(CN)_5NO$] • $2H_2O$), which is used as an exogenous source of NO. This drug acts as a potent peripheral vasodilator, influencing both arterioles and venules (venules in a stronger extent than arterioles). It is administered intravenously in cases of high blood pressure. The active component of the drug due to nitrosil metal complex contains 5 cyanide radical and a nitric oxide ligand. The iron atom is of second positive valence. Na-nitroprusside, released cyanide ions that in the liver are converted to thiocyanate under the influence of rodanidase (Butler, Megson, 2002).

The half-life of Na-Nitroprusside in the body is of the order of several minutes while cyanide ions, thiocyanate and thiosulphate remain in the body for hours and days. This means that some of the experimental data may be due to the combination of NO and other metabolic products such as cyanide or thiocyanate, especially at higher doses of the drug.

In our work we show experimentally the role of NO and other metabolic products of Na-nitroprusside (cyanide radicals, thiocyanates, thiosulphates) on the activity of AChE and BChE in various brain fractions.

This series was first traced the influence of Na-NP at concentrations 0,01-1,0 mM on the activity of AChE and BChE of the forebrain of white rats. The results of the study are presented as the activity of the enzyme (Fig. 3) as percentage of the control.

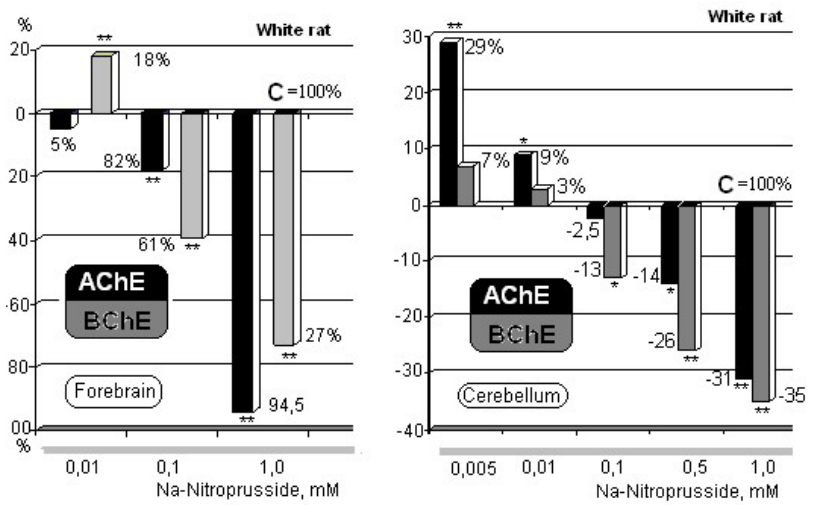

Figure 3. Influence of Na-Nitroprusside (0,005, 0,01, 0,1, 0,5 and 1,0 mM) as source of NO on the activity of AChE and BChE in the membrane-mitochondrial fractions of the forebrain and cerebellum of the white rat brain – percent relative to the control (C=100%)

The first conclusion of this study is that Na-Nitroprusside induced biphasic effect – stimulation at very low concentrations and concentration-dependent inhibition of both enzyme forms. In a later phase this agent (respectively NO) is a "typical" anticholinesterase agent. Such error was allowed by some authors, who suggested that Na-Nitroprusside (or NO) inhibits the AchE activity (Butler and Megson, 2002).

As shown, concentrations of Na-Nitroprusside (0,01-1,0 mM) are much lower than those in the series with L-Arginine (1,0-50 mM). This is due to a very complex structure of Na-Nitroprusside, which is metabolized to several products as NO, metal cyanide radicals, thiocyanates, thiosulphates and others (Shkodrova et al., 2013).

The stimulating action of Na-Nitroprusside on AChE and BChE activities in fractions of different brain areas of the brain of white rat occurred only at very low concentrations of the agent (0.005 to 1.0 mM). We believe that this is due to the NO, which is secreted into the fraction under the influence of nitric oxide synthase. A similar opinion was expressed by our colleagues, who studied the influence of Na-Nitroprusside on the activity of the mitochondrial ATPase of rat liver. For this purpose they used a special cell with two compartments, separated by a Teflon membrane, that allow the passing only of NO (Shkodrova et al., 2013). Stimulating esterase activity at low concentrations of Na-Nitroprusside (0.005 to 0.01) is better expressed in the enzyme activity in the cerebellum.

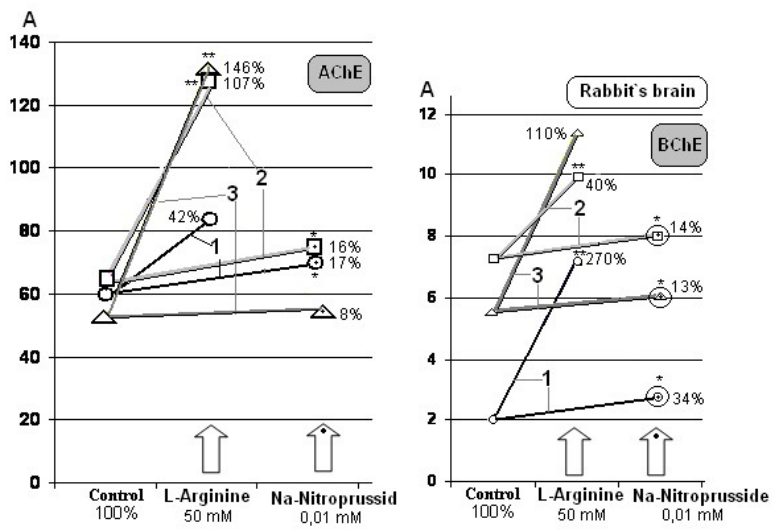

Figure 4. Influence of L-Arginine (50 mM) and Na-Nitroprusside (0,01 mM) as sources of NO on the activity of AChE and BChE in the membrane-mitochondrial fractions of the cerebral cortex (1), brain stem (2) and cerebellum (3) of the brain of rabbit
A – enzyme activity; n= 8-12; *p < 0.05; **p < 0.001

In parallel with the increase in the concentration of the reagent (0,1-1,0 mM) both enzymes were inhibited. This effect was also tissue specific. This inhibition of the enzymatic activity is undoubtedly due to other metabolic products of Na-Nitroprusside.

The influence of Na-Nitroprusside on the activity of AChE and BChe in three parts of the brain of a rabbit – brain cortex, brainstem and cerebellum (Fig. 4) was also studied. The maximum activation of both enzymes was at concentrations of 50 mM for L-Arginine and 0.1 mM for Na-Nitroprusside.

The data here show that L-Arginine greatly stimulates the activity of AChE in the cerebellum and brainstem (100%-150%), while this effect on the cerebral cortex (acetylcholine receptive structure) is only 42%. The effect of activation of Na-Nitroprusside (0,01mM) on AChE activity is within the lowest accuracy (8,0%-17%), which effect is best expressed in fractions of the cortex.

The pattern of activation of BchE activity is different. L-Arginine stimulates most of the enzyme fractions of the cortex (270%), and least in the brainstem (40%). There is a general pattern – the stimulation of enzyme activity is strongest where the starting activity is lowest.

The incentive effect of the Na-Nitroprusside (0,01 mM) on BChE activity is highest at fractions of the brain cortex (about 34%).

Finally, we considered that the activating efficiency of Na-Nitroprusside and L-Arginine due to NO (respectively by stimulating the activity of nitric oxide synthase), and the inhibition of enzyme activity by Na-Nitroprusside depends on other metabolic products listed above.

This conclusion is also confirmed by another experimental series, where the influence of the L-Arginine and Na-Nitroprusside (0.1- 10 mM) on the AChE activity of purified enzyme preparation of the torpedo fish (Fig. 5) was examined. This preparation does not contain NO synthase and only membrane-bound AChE.

The data show that L-Arginine at a wide concentration range does not affect the AChE activity. The effects of Na-Nitroprusside exhibit only inhibition of enzyme activity in parallel with the increase in the concentration of the reagent. At a concentration of 10 mM enzymatic activity was blocked almost 100%.

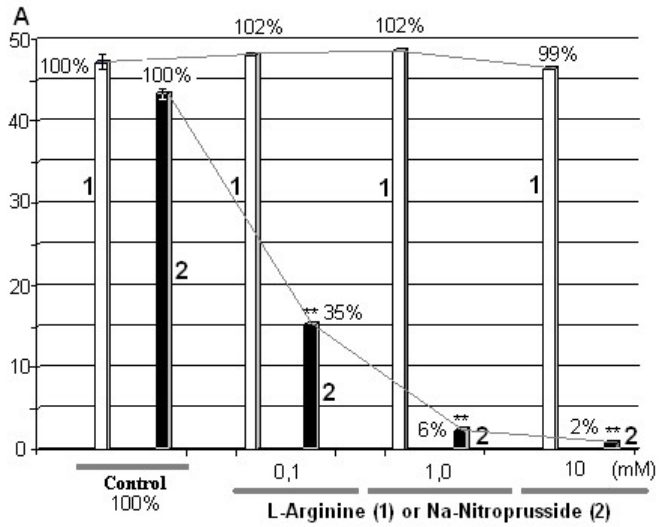

Figure 5. Influence of L-Arginine and Na-Nitroprusside in 0,1-10 mM concentrations on the activity of highly purified AChE from electric organ of the torpedo (Torpedo mormorata)

A - enzyme activity; n=8; ±m=1,50-3,60; *-p<0,05,**-p< 0,001

4. L-Arginine (NO respectively) as antidote for poisoning by certain anticholinesterase agents AChE and BChE are key components of the cholinergic system. These enzymes control synaptic activity in neural circuits and innervated organs, adaptation of organisms, their resistance against a lot of endogenous metabolites, poisons of plant and animal origin, chemical warfare agents, pharmaceuticals, synthetic pesticides and others. Genetic changes in the structure and function of these enzymes, as well as the influence of various damaging factors lead to serious nervous disorders and psychiatric diseases (Alzheimer's disease, neurosis, amnesia, paralysis and paresis, myasthenia, different syndromes and others).

The main principles of prevention and clinical intervention in cases of poisoning by anticholinesterase agents, such as organophosphates, carbamates, chemical warfare agents and others refer to acetylcholine receptors blockage and application in drug rapid metabolism of toxins.

In our study, we propose a new approach to treat poisoning by organophosphate and carbamate pesticides through preventive and clinical control of the cholinergic system, in particular the AChE and BChE.

This approach conventionally defines the enzymatic regulation as an antidote.

Firstly, we will make a brief analysis of the concentration-inhibition curve of AChE activity under the influence of specific blocking of this enzyme by carbamate (Fig. 6).

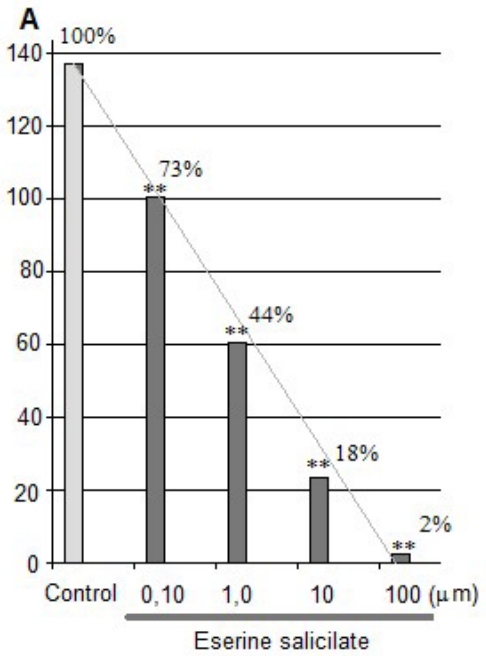

Figure 6. Influence of Eserine salicilate (0,10-100 μM) on the AChE activity of the cerebral cortex of the rabbit`s brain

A – enzyme activity; n=10; ±m=3,50-5,50; **-p< 0,001

Tabular form data

Control (C)	Eserine salicylate, μM			
	0,10	1,0	10	100
		A (Enzyme activity)		
136,95	100,43	60,26	23,50	2,30
		% relative to C		
100	73	44	18	2

The survey data strongly suggest the presence of concentration-dependent inhibition (CDI) of enzyme activity in this fraction. We believe this is a valid common law in such systems. Like the standard index DL50 (50% mortality of individuals in poisoning with a certain toxic agents in vivo), we introduce the index - CDI50 or CDS50 (concentration-dependent stimulation) by different agent in vitro. In our case CDI50 ratio is in the range of 1,0 μM eserine.

Our research shows that L-Arginine, respectively, nitric oxide, and other NO donors are modulators of the activity of AChE and BChE and can be used as antidotes in poisoning with organophosphate and carbamate pesticides.

The next step in the algorithm of the study is to follow the combined effects of eserine (in conc. of 10 μM) and L-Arginine (in conc. 50 mM). Our assumption was that L-Arginine will prevent the partial or complete inhibition, caused by eserine (Fig. 7).

Experimental data show that eserine salicylate (10 μM) inhibits about 80% of the AChE activity in the brain cortex of rabbit. Perhaps this effect in other brain fractions and species will be slightly different.

L-Arginine at 50 mM stimulates the activity of AChE by 96 %. A combination of eserine salicylate (10 μM) and L-Arginine (50 mM) firstly removes the inhibition caused by eserine, and second, L-Arginine in this combination stimulates AChE compared to control.

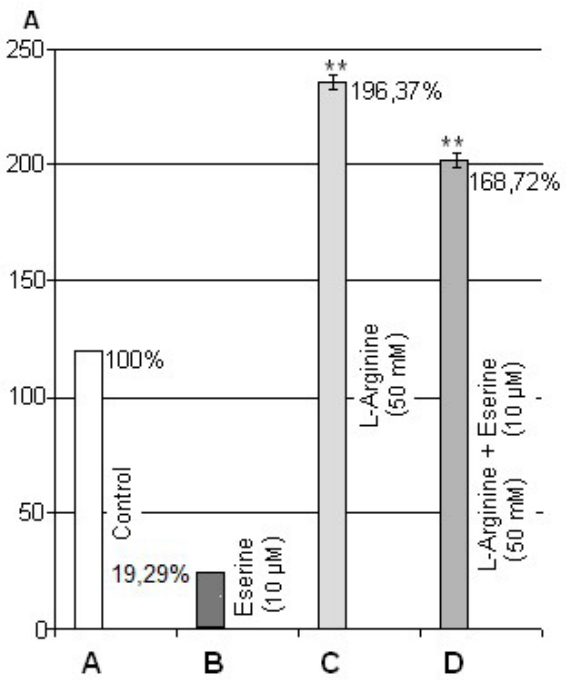

Figure 7. Role of L-Arginine respectively nitrogen oxide as a contender for the antidote for poisoning by carbamate pesticides. Influence of eserine salicylate (10 μM) (B), L-arginine (50 μM) (C) and the combination of eserine (10 μM) and L-Arginine (50 mM) (D) of AChE in the mitochondrial-membrane preparation of brain cortex of rabbit

A - Activity of the acetylcholinesterase (μg hydrolyzed acetylcholine/mg protein/ min); n = 15; ** - ρ <0,001

Tabular form data

Control	Eserine 10 μM	L-Arginine 50 mM	L-Arginine (50 mM) + Eserine 10 μM	
120	23,15	235,64	202,46	A
100%	19,29%	196,37%	168,72%	%

These facts clearly show that L-Arginine and other sources of NO restored normal output and activity of AChE. Thus, NO donors could counteract neurotoxic poisons.

CONCLUSIONS

Data on the influence of some endogenous (L-Arginine) and exogenous (Na-Nitroprusside) NO donors and their influence on the AChE and BchE activity were presented.

It was found that these substances stimulate considerably the activity of both enzymes. The level of activation depends on the histological structure, respectively, of the physiological rationale behind these brain structures.

The biphasic action of Na-Nitroprusside on the cholinesterase activity – stimulation at very low concentrations, caused by NO and concentration-dependent inhibition caused by other components of this agent was analyzed.

Our research shows the use of L-Arginine as an antidote against poisoning by organophosphorus and carbamate pesticides, chemical warfare gases and others poisonous substances. The competitive relationship between L-Arginine and eserine salicylate on the activity of AChE is a possible reason for this. L-Arginine in a wide concentration range dose-dependently removed inhibition, caused by anticholinesterase agents.

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This article is an expression of commitment to the ideas and activities of the Organisation for the Prohibition of Chemical Weapons, awarded to the Nobel Peace Prize in 2013 for their "extensive work to eliminate chemical weapons" (Cowel, 2013). Our study is also a small contribution in this direction.

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