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Effectiveness of L-arginine or nitric oxide as a reactivator and an antidote against cholinesterase pesticides and other toxic substances

Radoy I. Ivanov[1], Elitsa T. Dencheva[2]

[1]Sofia University "St. Kliment Ohridski", Faculty of Biology, Department of Animal and Human Physiology, Sofia, Bulgaria, radoy_011@abv.bg
[2]Sofia University "St. Kliment Ohridski", Faculty of Biology, Department of Zoology and Anthropology, Sofia, Bulgaria, elidencheva@abv.bg

Abstract

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The cholinergic system is one of the most important neuromediator systems in organisms that manage physiological functions, biology, behavior, adaptation, intellect. Cholinesterases (AChE and BChE) occupy an important place in the structure of this system. AChE participates in the mechanisms of synaptic conduction, while BChE mainly hydrolyses many toxic products that damage the nervous system. The main objective of this study was to establish the levels of stimulation on the activity of cholinesterases by some donors of nitric oxide (for example L-arginine) and to estimate the role of nitric oxide (NO) as a mediator that controls the activity of these enzymes. Another objective of the study to obtain data useful in biomonitoring assessment of ecotoxicology environment and creation of new approaches for prevention and therapy against specific intoxicants and pesticides. The main finding of the study is the revealed significant activation of cholinesterases from L-arginine (10-50 mM) in various brain structures in mammals (*Rattus rattus* and *Oryctolagus cuniculus*) and in invertebrate fractions from *Vespula germanica* and *Apis mellifera*. This stimulation is better demonstrated for BChE, which can reach eight times in magnitude; L-arginine, as donor of NO, plays an importante role as a protector and reactivator of the inhibited activity of cholinesterases and as an antidote for severe intoxications from pesticides and other toxic products, including cyanides; the application of a broad spectrum NO synthases inhibitor (L-NMMA) removes partially or fully activates the activity of cholinesterase in fractions of vertebrates and invertebrates with added toxins. This means that NO is an important factor in the control of cholinesterase activity.

Keywords: cholinesterase activity; L-arginine; nitric oxide; pesticides; ecotoxicology; antidote; L-NMMA

Introduction

In previous papers (Ivanov and Dencheva, 2016; Dencheva and Ivanov, 2017) we reported data on the effects of two NO donors – L-Arginine and Na-nitroprusside on the activity of AChE and/or BChE in different fractions of vertebrates and invertebrates. Cholinesterases are important components of the cholinergic system, which manages almost all physiological processes and the behavior of organisms (Ivanov, 2006, 2008). Here, it is appropriate to mention the different basic functions of the two types of cholinesterases – synaptic efficacy (AChE) and the prevention of the nervous system against toxic products (BChE). One of the major problems in ecology is the pollution with various pesticides, some of them with anticholinesterase activity (Čolović et al., 2013), as well as with esters of narcotics (Zheng et al., 2008; Chen et al., 2015) or other poisons of different origin, which are mainly degraded by BChE (Pohanka, 2013). These and other data and the results of our studies indicate the possibility of using of NO donors as reactivators to cholinesterase activity.

This article presents new data for possible application of L-Arginine as a detoxifying agent against specific toxic products. One of the important issues in this aspect is about the molecular mechanisms of activating efficacy of L-arginine-like products. In this work the emphasis is on the role of NO in the regulation, modulation and reactivation of cholinesterase activity. This suggestion was verified using of a broad spectrum NOS-inhibitor L-NMMA (L-N^G-monomethyl Arginine) (Taylor, 1997; Stuehr, 1999; Cotter et al., 2000; Liu and Feng, 2012).

Material and Methods

Preparation of enzyme fractions. Isolation of membrane and mitochondrial fractions of brain of two mamalian species – Wistar rat (*Rattus rattus*) and rabbit (*Oryctolagus cuniculus*) as well as from *Vespula germanica* and *Apis mellifera* was performed by the method of differential centrifugation. The medium for homogenization and isolation of the respective fractions is in the composition of 0.1 M KCl, 1 mM $MgCl_2$, 0.1 mM EDTA-Na salt, 50 mM Tris-HCl (pH 7.6) (Ivanov and Dencheva, 2016; Dencheva and Ivanov, 2017). The animals used in the laboratory tests are from the vivarium of the Faculty of Biology, Sofia University and invertebrates – from natural sources. The investigations conformed to the international and national rules and regulations requirements for ethical attitude towards the animals.

Determination of acetylcholinesterase and butyrylcholinesterase activity. There are many methods for determination of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity. We measured AChE and BChE activities by classical method of Ellman et al. (1961). The activity of AChE and BChE was determined depending on the thiocholine (ThCh) released during the hydrolysis of acetyl thiocholin iodide (AThChI) and butyryl thiocholin iodide (BThChI). The thiocolline content was measured spectrophotometrically at an optical density at l = 412 nm. A reaction was stopped with adding the specific blocker of AChE or BChE – Eserine salicylate (100–500 μM).

Calculation of enzyme activity. The principle of measurement of cholinesterase activity is measurement of contents of SH-group. Therefore, we used L-cysteine ($C_3H_7NO_2S$) in different molar concentration to prepare a standard curve. The molar content of SH-groups in cysteine and thiocholine (ThCh) is the same. The SH-groups of ThCh reacts with dithionitrobensonic acid (DTNB) which is reduced in a yellow-colored product – thionitrobenzoic acid (mercaptonitrobenzoate; MNB) that was spectrophotometrically measured. According to the methodology for calculating the enzymatic activity and to the data from the standard curve it is necessary to calculate the recalculation factor (k) of the enzymatic activity. The enzymatic activity of the respective fractions in the article is presented in real values (A) in μg or μM hydrolyzed substrate/mg protein/min, in relative units (r.u.) or in % relative to the respective controls.

Determination of content of the protein. The protein content in the samples was determined by the classical method of Lowry et al. (1951). For this purpose, lyophilized albumin (10-100 mg/ml) was used for standard curve preparation. The spectrofotometrical density (E) of the samples was determined at λ = 750 nm. According to data of this calibration curve was calculated conversion factor (k) (coefficient for recalculation).

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

Results and Discussion

I. L-Arginine as activator of AChE and BChE in different fractions of invertebrates and vertebrates

In this section data are presented on the activating effectiveness of L-arginine on AChE and BChE from various tissues and species of animals – vertebrates (*R. rattus*, *O. Cuniculus*) and invertebrates (*A. mellifera*, *V. germanica*). The aim was to compare the effects of L-Arginine in different tissues and species, to track the localization of cholinergic elements in certain areas of the body and to develop technology for protection of organisms agains intoxication.

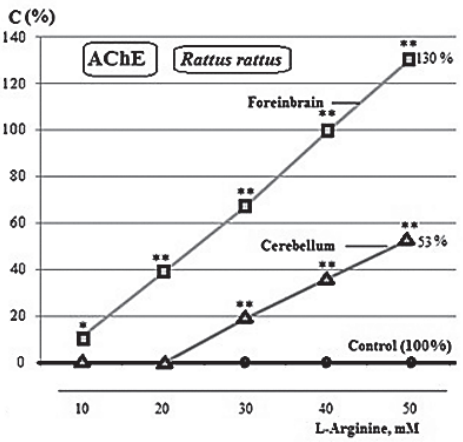

Fig. 1. Influence of L-Arginine (10-50 mM) on the activity of AChE and BChE in fractions of the forebrain and cerebellum in the rat's brains (*R. rattus*)

C (%) – % relative to the control; n = 8-12; ±m = 3.0-5.0; * – $p < 0.05$; ** – $p < 0.001$

The influence of L-Arginine on the activity of cholinesterases in different brain regions (Fig. 1-3) can be described as concentration-dependent stimulation of enzyme activity. This efficiency is different in various brain structures. The main reason for this difference is the distribution, the structure and functions of neurons in the relevant topographic zones. Such differences appear also in invertebrates.

Other interesting fact is that cholinesterases (AChE and BChE) in the foreinbrain of a Wistar rat (Fig. 1, 2) are much more sensitive to L-Arginine than its cerebellum. This was demonstrated by levels of activation of enzyme activity (e.g., 130% in the foreinbrain and about 50% in the cerebellum for AChE). The initial effective brain concentration for both enzymes is about 15-25 mM higher than that in the forebrain; the very high level of BChE stimulation in the rat`s forebrain (about 8 times) is noticeable (Fig. 2).

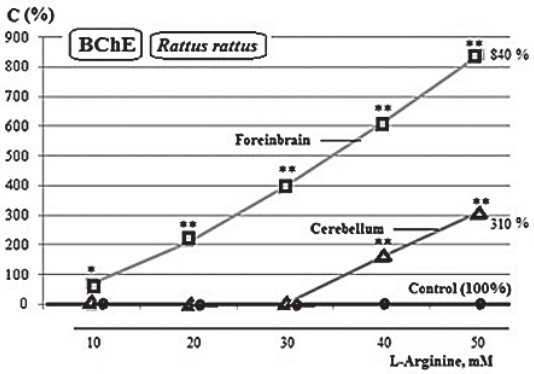

Fig. 2. Influence of L-Arginine (10-50 mM) on the activity of BChE in fractions of the forebrain and cerebellum in the rat's brain (*R. rattus*)

C (%) – % relative to the control; n = 10; * – $p < 0.05$; ** – $p < 0.001$

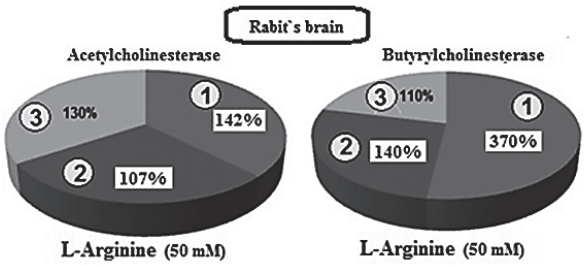

Fig. 3. Influence of L-Arginine (50 mM) on the activity of AChE and BChE in fractions of brain cortex (1), brain steam (2) and cerebellum (3) of the rabbit's brain (*O. cuniculus*)

The data are in % relative to the control; n = 8-10

Different results and trends of influence of L-Arginine were obtained in total fractions of some invertebrates. Thus, L-Arginine stimulates the activity of AChE only in European honeybee, but does not affect the enzyme activity of German Wasp (Fig. 4).

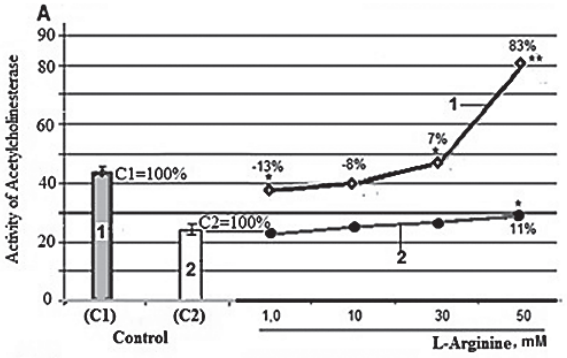

Fig. 4. Influence of L-Arginine (1,0-50 mM) on the activity of AChE in fractions of *A. mellifera* (1) and *V. germanica* (2)

A – activity of the AChE (μg hydrolyzed acetylcholine/mg protein/min); n = 8-10; ±m = 3.5-5.0; * – $p < 0.05$; ** – $p < 0.001$

II. L-Arginine as a reactivator of inhibited cholinesterase in invertebrates and vertebrates and as antidote for poisoning against choline esters and other toxic products

About 30% of insecticides used in agricultural practice exert their toxic action by specifically suppressing of cholinesterase activity. Carbamates are organic compounds derived from carbamic acid (NH_2COOH). In the study, predominantly natural carbamates – eserin (physostigmine) were used. Carbamate pesticides inhibit reversibly cholinesterases in vertebrates and invertebrates. Some of the important insecticides of the carbamate line are albicarb, carbofuran, carbaril (Sevin), fenobucarb, oxamil and others (Fukuto, 1990). Some carbamates (icaridin) play the role of insect repellent. Others (aldicarb, carbofuran) are highly toxic for humans and were tested for military use as nerve agents (Ellison, 2008; Gupta, 2015).

In our previous studies (Ivanov and Dencheva, 2016; Dencheva and Ivanov, 2017), it has been shown that L-Arginine specifically affects the activity of cholinesterases in various tissues and animal species. This influence is mainly stimulating the enzymatic activity, which in some species of vertebrates is very strongly manifested on BChE. As it is known, this enzyme type is a "firewall" against any endogenous and exogenous metabolites and toxic products that damage the nervous system (choline esters, medicinal prod-

ucts, narcotic agents, pesticides and insecticides, warfare gases, some forms of snake poison, etc.). The phenomenon of genetic BChE deficiency in humans is characterised with very high sensitivity and intoxication to certain poisonous products. This study shows the possibility of detoxification of organisms through a significant increase of BChE activity. In the literature there are sufficient evidences that about 95% of heroin or cocaine in the body is hydrolyzed by BChE (Hidaka, 1997; Browne et al., 1998; Barta et al., 2001; Zheng et al., 2008; Pohanka, 2013; Chen et al., 2015).

One of the ideas is that the activating effectiveness of NO donors on cholinesterases depends on the released NO. In our project such sources are L-Arginine and Na-Nitroprusside. The effects of these reagents, however, are different in nature. This is due to the differences in their molecular structure. For example, Na-Nitroprusside in an aqueous medium dissociates into the molecule NO, 5 cyanide ions and the ferricyanide complex. Cyanide radicals and perhaps the ferricyanide complex effectively and concentration-dependently inhibit cholinesterases. In this situation, the effects of Na-Nitroprusside resemble those of specific anticholinesterases, but at a much higher concentration (Friederich and Butterworth, 1995) (Fig 5).

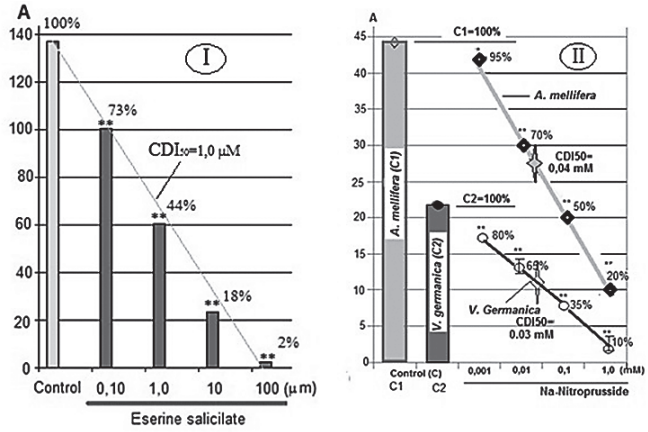

Fig. 5. Influence of Eserine salicilate (0,10-100 μM on the AChE activity of the cerebral cortex of the rabbit's brain) (I) and the role of Na-Nitroprusside on the AChE activity in the fraction of *A. mellifera* and *V. germanica* (II)

A – Activity of the AChE (μg hydrolyzed acetylcholine/mg protein/min); n = 15; ±m = 3.5-5.5; * – p < 0.05; ** – p < 0.001

The data from Fig. 5 show several important relations: concentration-dependent inhibition of AChE by both specific anticholinesterase agents and ferricyanide products (Čolović et al., 2013) in both invertebrates and vertebrates; the total effect of Na-Nitroprusside on the activity of AChE is inhibition, although this preparation releases NO; the toxicity (according to the CDI_{50} coefficients) of the cyanide compounds is many times lower than that of serine; the resistance of AChE in fraction of *V. germanica* to ferricyanide radicals is about 1.3 times lower than that of *A. mellifera* (Hamel, 2011; Anseeuw et al., 2013).

One of the objectives of the study is to reveal the role of L-Arginine as reactivator of AChE activity, suppressed by carbamate pesticides and cyanides in various animal fractions and species. Details of this are presented in Fig. 6, where the role of L-Arginine as reactivator of the AChE activity is shown for brain cortex of rabbit brain (I) and for *A. mellifera* fractions (II). These data strongly suggest that L-Arginine (50 mM) completely eliminates the inhibition of enzyme activity induced by a specific carbamate pesticide. Similar but partial is the effect of 30 mM L-Arginine 30 mM.

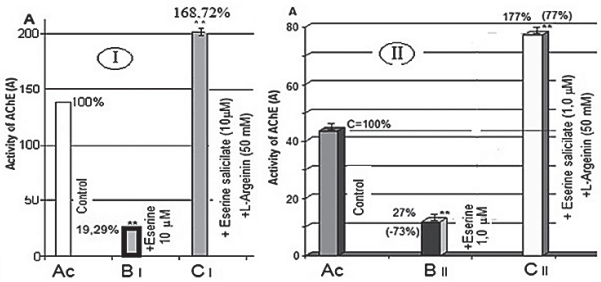

Fig. 6. L-Arginine (50 mM) as a reactivator for the suppressive activity of AChE from Eserine salicylate (B_I or B_{II}) in rabbit`s brain cortex (I) or in A. mellifera (II) fractions. The reactive efficacy of L-Arginine is expressed in combination with the corresponding concentrations of Eserine.

A – Enzyme activity in μg hydrolysed AChE/mg protein/min; n = 10; ±m = 4.0-5.5; ** p<0.001; Ac – control

This also means that L-Arginine or secreted NO can be used as an antidote for intoxication with anticholinesterase agents or cyanides. According to the literature and our research data, L-Arginine can be used to treat overdoses of drugs as cocaine and heroin, and against poisoning with various toxic products, as well as to save beehive families from specific organophosphates, carbamates, neonicotinoids and other pesticides and insecticides (Tapiero et al., 2002; Morris, 2004).

III. Nitric oxide as a factor for control the activity of cholinesterases

In this final part we aimed to reveal some components of the mechanism of activating influence of NO donor L-Arginine on chlolinesterase activities in the studied animals. The

data provided here suggest that in most cases L-Arginine significantly stimulates cholinesterases in various fractions of vertebrate and invertebrate animals. Such stimulation was not found only in *V. germanica*. It was mentioned that this was mainly due to low NOS activity, i.e. the limited endogenous NO release. In all other cases, the addition of L-NMMA caused a sharp decrease in the L-arginine stimulating effect. A typical example for the other studied species – *A. mellifera* – is presented in Fig. 7.

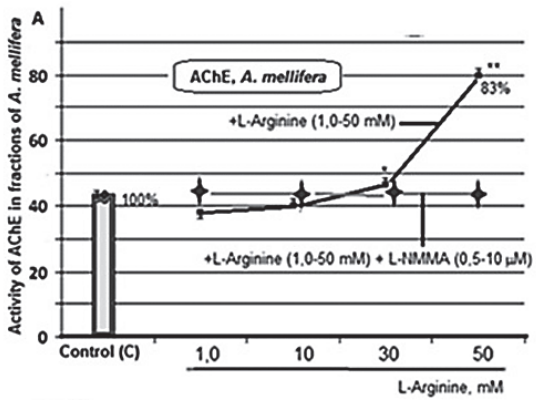

Fig. 7. Influence of L-Arginine (1.0-50 mM) and the combination of L-Arginine and L-NMMA (10 µM) on the activity of AChE in *A. mellifera* fractions

A – Activity of the AChE (µg hydrolyzed acetylcholine/mg protein/min); n = 10; ±m = 4.0-5.5; * – p < 0.05; ** – p < 0.001

It is evident (Fig. 7) that L-Arginine in concentrations from 10 to 50 mM concentration-dependently activates AChE in *A. mellifera* fractions (about 80% under the influence of L-Arginine concentration of 50 mM). According to these data, the series with added L-NMMA (10 µM) nearly completely eliminate the activating effect of L-Arginine. This result suggests the participation of NO in the stimulatory action of L-arginine on animal cholinesterases, in this particular case of AChE in some invertebrates. Similar effect of L-NMMA on the activity of AChE is observed in fractions of rabbit`s brain cortex (Fig. 8).

The combination of L-Arginine and L-NMMA almost fully suppressed the activating action of L-Arginine and the differences between the test and control values are not credible. L-NMMA in concentration about 10 M removes the activating effect of L-Arginine on cholinesterases also in other studied animal structures and species.

This suggests that the main if not the only mechanism for explaining the activation of cholinesterases by some NO

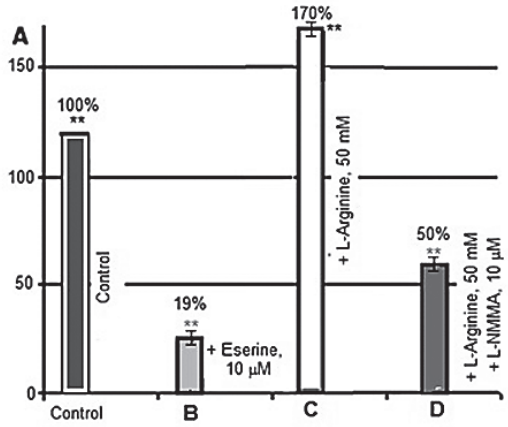

Fig. 8. Effect of Ezerine salicylate (10 µM) (B), L-Arginine (50 mM) (C) and combination Eserine (10 µM), L-Arginine (50 mM) and L-NMMA (10 µM) (D) on the activity of AChE in rabbit's cerebral brain cortex

A – Enzyme activity in µg hydrolysed ACh/mg protein/min; % – % relative to the control; n = 8-10; ± m = 4.0-5.5; ** p < 0.001

donors is mediated by NOS-dependent NO production. One of the proofs of this is the complete or partial removal of the activating efficacy of, for example, L-Arginine in blocking the NOS activity of L-NMMA. Another fact in this sense is the lack of activation of highly purified AChE from electrical organs of some electric fish by L-Arg (Ivanov and Dencheva, 2016).

Conclusions

The results of the study show for the first time that L-Arginine and some other NO donors stimulate, regulate or modulate the activity of cholinesterases in certain invertebrate and vertebrate species. This activation is tissue and species specific. It depends on the presence of cholinergic elements in the respective fraction.

In different areas of mammalian brain the activation of BChE is stronger. This fact suggests the possibility of using of L-Arginine as a reactivator of inhibited AChE activity in a toxic environment. This is a new method for protection of organisms and humans from toxic products – anticholinesterase agents, narcotic substances (cocaine, heroin, etc.), medicinal products and against chemical neuropathic gases gases (for example, sarine).

This new way of detoxification is different from the protection of acetylcholine receptors like atropine, scopolamine, hexamethonium, etc. (Robenshtok et al., 2002; Marrs, 2003), or via oxime reactivators like pralidoxime,

obidoxime, etc. (Sogorb and Vilanova, 2002; Eyer, 2003; Bajgar et al., 2007). It is focused on enzymatic degradation of acetylcholine and other toxic products from AChE and/or BChE, respectively stimulated by corresponding donors of NO (for example, L-Arginine). It may be suggested that combining these approaches for prevention and therapy based on control of cholinesterase activity will give a much better result.

The main goal of the studt was to find rapid and effective technology for evaluation, prevention and therapeutics against intoxications of organisms with products manifesting anticholinesterase activity. Here we provide evidences that one of the main regulators of cholinesterases in the norm and poisoning is NO. This has been achieved by the use of a broad-spectrum NOS inhibitor L-NMMA (Kellogg et al., 2005).

Acknowledgments

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