Bulletin of High Institute of Public Health
Vol. 37 No. 2 [2007]

Effect of the Carbamate (Physostigmine) on the Activity of Nitric Oxide Synthase in Different Parts of Rat Brain (Invivo Studies)

Hassan M.Y Osman*

Abstract: Physostigmine (eserine) is the methyl carbamic ester of phenolic trimethyl ammonium compound. It is a powerful inhibitor of acetylcholinesterase enzyme activity and used in some therapeutic preparation. In this work, experiments were carried out in-vivo to study the intraperitoneal (i.p.) infusion of eserine on the activity of nitric oxide synthase (NOS) enzyme, obtained from whole and five different parts of rat brain, namely: basal ganglia, frontal cortex, medulla oblongata, pons, and cerebellum. In this work ,two experiments were carried out. Experiment A: to study the dose dependence of i.p. infusion of eserine on NOS activity. Experiment B: to study the time dependence post-infusion of constant dose of eserine (the dose which caused 50% inhibition of the enzyme activity: I_{50}). The results showed that the inhibition of the enzyme occurred in each part studied, and the inhibition increased with increasing the infused dose of eserine, and the time post i.p. infusion, i.e., the inhibition is dose and time dependent. The highest inhibition occurred in the pons and medulla oblongata extracts; these parts are responsible for the reflex centers of cough and vital centers.

Key words: Physostigmine; Carbamate; Nitric Oxide Synthase

INTRODUCTION

Carbamate pesticides poisoning tends to be less severe than organophosphorus pesticides (OP) because they bind reversibly to the active site of the enzyme, in contrast to OP pesticide that binds irreversibly.(1) Carbamate pesticides inhibit rat brain nitric oxide synthase (NOS) activity through inhibiting the regulatory protein calmodulin (CaM) (NOS stimulator) activity without affecting the basal enzyme activity; leading to brain toxicity.(2)

Nitric oxide (NO) is a regulatory biological substance and an important intracellular messenger that acts as a specific mediator of various neuropathological disorders.(3) NO plays an

*Biochemistry Department, Medical Research Institute, Alexandria University

important role as a neurotransmitter in the nervous system, as vasodilator in the cardiovascular system and as a cytotoxin in the host defence mechanism of macrophages.[4] Nitric Oxide Synthase (NOS) was first identified and described in 1989. The demonstration of the formation of NO by an enzyme (NOS) in vascular endothelial cells opened up what can be considered a new area of biological research.[5] Brain NOS is a constitutive enzyme and the function of which is to produce NO on demand for diverse neurophysiological activities.[6] Physostigmine (eserine) is a tertiary nitrogen atom derivative which blocks conduction and enzyme activity.[7]

The aim of this work is to study the *in-vivo* effect of i.p. injection of the carbamate eserine on the activity of nitric oxide synthase (NOS) enzyme obtained from whole and five different parts of rat brain namely: basal ganglia, frontal cortex, medulla oblongata, pons, and cerebellum, and to know which of these parts is most affected by eserine. Also, the present study aimed at indicating the inhibitory power of eserine on NOS enzyme on one hand and which parts of the brain was inhibited than the others on the other hand.

MATERIAL AND METHODS

Fifty male albino rats (125-150 gram body weight) age 2 months were used in the experiments. Rats were supplied from Medical Research Institute Animal House, Alexandria University (Egypt) and were housed in group cages (five in each) and given free access of food and tap water (ad libitum). The brains of these rats were used as a source for NOS. The rats were killed by decapitation after subjected to an overnight fast, with free access of water. Each brain weighed about 1.6 g.

Chemicals used were:

Physostigmine (eserine) from Aldrich Chemical Company Co. Ltd, Gillingham (England), Naphthylethelenediamine dihydrochloride, Sigma (Deisonhofen Germany), Sulfanylamide, Sigma

Deisonhofen Germany, Phosphoric acid (H_3P0_4): Deisonhofen Germany and Buffer: Phosphate (KH_2PO_4 and Na_2HPO_4) 0.1 M, pH 8.0 BDH.

Experiment A : (Dependence on the dose of the inhibitor):

25 rats were used in this experiment. The control group (5 rats) were injected i.p. with saline, while each experimental group (5 rats each) were injected i.p. with eserine in doses of 1.0, 2.0, 3.0, and 4.0 mg/kg body weight. The rats were decapitated 9 minutes post- injection. At this time, signs of tremors, shivering, tachypnea, and craving for air appeared on the rats and most of them died 15 min. post infusion of 4.0 mg eserine/Kg BW.

Experiment B: (Dependence on the time course post injection).

25 rats were used in this experiment. The control group (5 rats) were injected i.p. with saline as experiment A, while the experimental groups (5 rats each) were injected with a constant dose 3.0 mg/kg body weight (I_{50}) of eserine. The rats were decapitated at various time interval (3, 6, 9, and 12 min).

In the above experiments A as well as B the whole as well as the different parts of the brain were isolated, washed with ice cold saline and weighed each separately. These parts are frontal cortex (15 g), basal ganglia (8 g), pons (7 g), medulla oblongata (10 g), and cerebellum (15 g). The parts were homogenized in ice cold phosphate buffer Na2HPO4: NaH2PO4, pH 7.4, 0.1 mole/L and used for the enzyme assay.

Assay of Nitric oxide synthase (NOS) enzyme activity:

Brain Nitric oxide synthase (NOS) is a constitutive enzyme and the function of which is to produce nitric oxide (NO).

Nitric oxide (NO) is also a neurotransmitter formed in addition to citrulline from L-arginine through mediation of the enzyme nitric oxide synthase (NOS).

$$\text{L-Arginine} \xrightarrow{\text{NOS}} \text{L-citrulline} + \text{NO}$$

NOS was determined by estimating nitrite. Nitrite was being the most stable metabolite of oxide. It is used as an index for NOS.[8,9]

Due to the transient and volatile nature of NO which makes it unstable, NO were oxidized to the stable compounds nitrite (NO_2) and nitrate (NO_3). The most convenient detection method for NO was measured in term of NO_2 which can be detected by photometric method using Griess reagent(8) at 546 nm.

Griess reagent consists of one part of naphthylethelenediamine dihydrochloride (NED) in distilled water and one part of 1% sulfanilamide in 5% concentrated phosphoric acid (H_3PO_4). The two parts being mixed together within 12 h of use and kept chilled. Each part may be stored separately refrigerated up to 2 months. The mixture of the two parts is incubated with a NO_2-continaing sample (ratio 1:1) to form purple azo dye and its absorbance is measured at a wave length of 546 nm.(8,9)

To determine NO2 in brain tissue, 150 µl brain homogenate containing 20 mg tissue was mixed with 1.5 ml of Griess reagent and the mixture incubated for 5 min at room temperature, the absorbance was measured at 546 nm. Nitrite were calculated from NO_2 standard curve.

RESULTS

a) Dependence on the different doses of eserine (experiment A)

Table 1 indicates the activity of NOS in whole and five different parts of rat brain. It has been found that the basal ganglia possesses the highest activity (28 µmol of NO_2 produced/min/g wet wt of tissue, while the frontal cortex possessed the lowest activity (24 µmol of NO_2 produced/min/g wet wt of tissue). Table 1 also indicates the effect of eserine on NOS obtained from whole and five different parts of rat brain. The inhibition of NOS in the different parts increased with increasing the infused dose of eserine. Rats were killed after 9 min post

injection, due to the signs of tremors and creeping with their hind limbs at 3.0 mg/kg BW. The highest inhibition occurred in the pons and medulla oblongata extracts (Fig. 1), and the lowest in the cerebellum, basal ganglia and frontal cortex extracts.

b) Dependence on the different time post infusion of constant dose of eserine (experiment B)

Table 2 shows that the activity of NOS post injection of constant dose of eserine 3.0 mg/KgBw of rats. The highest inhibition occurred in the medulla oblongata and the pons extracts, (Fig. 2), while the lowest inhibition occurred in basal ganglia and frontal cortex extracts.

DISCUSSION

The experiments showed considerable difference in NOS activity in the various parts of rat brain. The highest activity was detected in the cerebellum, lower in basal ganglia, and the lowest in the frontal cortex. The high NOS activity in the cerebellum is in agreement with the results described by other authors.[10-13] In the present work, the i.p injection of different doses of the carbamate eserine resulted in the inhibition of NOS activity. Likewise, previous studies showed that the organochlorine and organophosphorus compounds owing to their lipophilic nature, bind to the hydrophobic region of CaM and thus impair CaM dependent NOS activities like Ca^{2+}ATPase phosphodiestrase.[13] Also, Roa *et al*.,[2] found that the Carbamate sevin inhibits rat brain nitric oxide synthase (NOS) activity through inhibiting the regulatory protein calmodulin (CaM) (NOS stimulator) activity without affecting the basal enzyme activity; leading to brain toxicity.

The present work showed that the inhibition of NOS activity decreased with increasing the concentrations of eserine, i.e., dose dependence. The NOS obtained from medulla oblongata as well as pons showed the highest inhibition while the lowest inhibition was obtained in the cerebellum, basal ganglia and frontal

cortex. This indicated that the eserine affects the NOS of pons and medulla oblongata more than other parts. In this respect eserine resembles sevin in its inhibition of rat brain NOS.[14]

Regarding the effect of time post injection of constant dose of eserine, the results indicated that the inhibition reached about 50% after 12 min post injection of 3.0 mg/KgBw eserine. This inhibition increased with increasing the time post injection, i.e., it is also time dependent.

It has been found that the results obtained from experiment B coincises with those obtained from experiment A. Complete inhibition of NOS wasn't obtained in any part of the brain through the 12-minute course post infusion in spite of the signs of tremors and suffocation of the rats.

Finally; it is note-worthy to mention that the difference in the rate of NOS inhibition by eserine in different parts of rat brain could be due to:

a. The inhibition NOS in the various regions of the brain is dependent on the concentration or the form of the enzyme, i.e., isoenzyme phenomenon.
b. The rate of supply of the inhibitor differs because blood flow differs between areas of the brain.
c. The high effect of eserine on NOS activity may be due to the solubility of this compound in the lipid layer in the brain as eserine is a tertiary nitrogen compound and affects the parts containing white matter more than the parts containing gray matter, as previously proved by Osman et al.[15]

- The parts which were the most affected with eserine were the pons and medulla oblongata (parts concerned with vital centers

Table 1: The effect of different dose of eserine on whole and different parts of rat brain nitric oxide synthase (NOS) at constant time (9 min). The activity of NOS expressed as µmol of nitrite (NO_2) produced/g of tissue/min.

Part of brain	Dose of infused eserine in mg / KgBw					% Inhibition at 4.0 mg/kg BW
	0*	1.0	2.0	3.0	4.0	
	NOS Activity					
Whole brain	31.50	27.00	22.50	18.70	16.40	48.00
Basal ganglia	28.00	25.50	22.00	16.00	15.50	44.64
Frontal cortex	24.00	23.00	18.50	12.50	13.00	45.80
Medulla oblongata	27.00	21.50	16.00	13.50	11.60	57.00
Pons	26.00	22.50	16.80	14.00	11.70	55.00
Cerebellum	34.00	31.00	28.00	23.50	21.00	38.20

*) Control

Figure (1): The effect of different doses of eserine on whole and five different parts of rat brain NOS at constant time (9min)

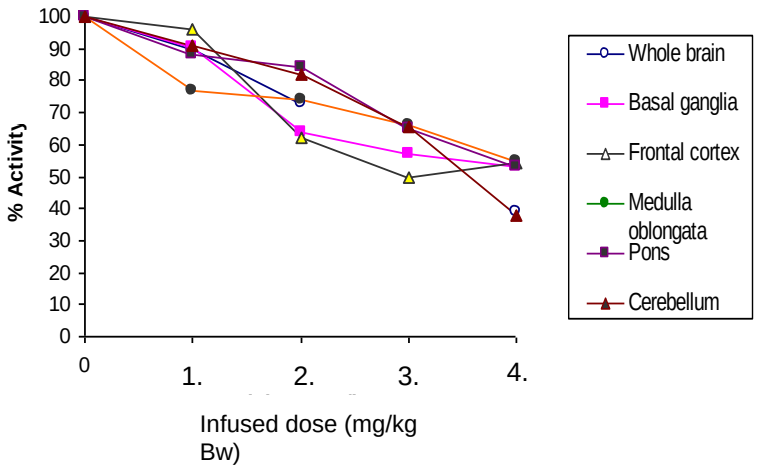
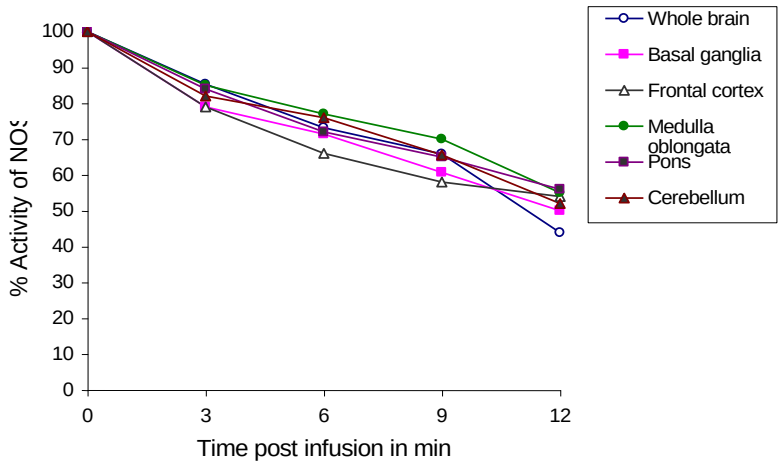


Table 2: The effect of constant dose of eserine (3.0 mg/KgBw) on whole and different parts of rat brain nitric oxide synthase (NOS) at various times post injection. The activity of NOS expressed as µmol of nitrite (NO2) produced/g of tissue/min.

Part of brain	Time post injection in min					% Inhibition at 12 min
	0 *	3	6	9	12	
	NOS activity					
Whole brain	31.50	28.00	21.50	16.40	15.00	52.40
Basal ganglia	28.00	22.00	20.00	15.50	14.50	48.20
Frontal cortex	24.00	19.00	16.00	13.00	12.00	50.00
Medulla oblongata	27.00	22.50	17.00	13.50	11.50	57.00
Pons	26.00	21.00	16.50	13.00	10.50	60.00
Cerebellum	34.00	28.00	26.00	21.00	18.50	45.60

*) control

Figure (2): The effect of constant dose of eserine (3.0mg/KgBW) on whole and five different parts of rat brain NOS at various time post injection



REFERENCES

1- Vidair CA. Age dependence of organophosphate and carbamate neurotoxicity in the postnatal rat: extrapolation to the human. Toxicol Appl Pharmacol. 2004; 2: 287-302.

2- Roa MR, Kanji VK, Sekhar V. Pesticides induced changes of nitric oxide synthase in rat brain invitro. Drug chem toxicol. 1999; 2: 41– 20.

3- Rodrigo J, Fernandez AP, Alonso D, Serrano J, Fernandez-Vizarra P, Martinez-Murillo R, *et al*. Nitric oxide in the rat cerebellum after hypoxia/ischemia. Cerebellum. 2004; 4: 194-203.

4- Bredt DS, Snyder SH. Nitric oxide mediated glutamated glutamate-linked enhancement of cGMP levels in the cerebellum . Proc Natl Acad Sci USA. 1989; 86: 9030-3.

5- Ktatt P, Schmidt K, Mayer B. Brain NOS is a hemoprotein. Biochem J. 1992; 288: 15-7.

6- Robbins PA, Hamel FG, Floreani AA. Bovine bronchial epithelial cells metabolize L-arginine to U-citrulline. Possible role of NOS. Life Sci. 1993; 52: 709-16.

7- Elliot KAC, Page I.H, Questel VH, (eds) Neurochemistry. The chemistry of brain and nerve. 2nd ed. p.544 Springfield. IL. USA: Ch. C. Thomas; 1962.544

8- Marzinzing AKM, Nussler J, Stadler E, Marzinzing W, Barthlen NC, Nussler HG, *et al*. Improved method to measure end product of NO in biological fluid. Nitric oxide. Biology and Chemistry. 1997; 1: 177-89.

9- Ayub S, Verma J, Das N. Effect of endosulfan and malathion on lipid peroxidation, nitrite and TNF-α release by rat peritoneal macrophages. International Immunopharmacology. 2003 ; 3: 1819–28.

10- Forstermann U, Gorsky LD, Pollock JS, Schmidt HH, Heller M, Murad F. Reginal distribution of EDRF/NO-synthesising enzyme (s) in rat brain. Biochem Biophys Res Commun. 1990; 119 : 727-32.

11- Kidd EJ, Michell AD, Humphrey PP. Autoradiographic distribution a 3H-L-NG nitroarginine binding in rat brain. Neuropharmacology. 1995; 34: 63-73.

12- Halacak L, Zigova Z, Klimova L, Bernatova I, Pechanova O. Inhibition of NO activity in nervous tissue leads to decreased motor activity in the rat. Physiol Res. 2000; 49: 143-9.

13- Barjavel MJ, Bhargava HN. Nitric oxide synthase activity in rat regions and spinal cord of mice and rats: Kinetic analysis. Pharmacology. 1995; 50: 168-74.

14- Nassif MM. Effect of two organophosphorous and two carbamate pesticides on the activities of acetylcholinesterase and nitric oxide synthase in different parts of rat brain. MSc. Thesis submitted to Medical Research Institute, Alex. Univ, Dept. of Applied Medical Chemistry. 2006; 28.

15- Osman MY, Abdel Tawab S, Sharaf IA. Effect of physostigmine on cholinesterase activity in different parts of rat brain. Arzeneim-Forsch. 1995; 45(1): 663-5.