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Original Article

Effect of Paraoxon on GABA Uptake by Rat Cerebral Cortex Synaptosomes

A Ghasemi[1], A. Sadidi,[2] A. Asgari,[1,3]
A. Khoshbaten[1,3]

Abstract

Background: It has been suggested that organophosphates may inhibit gamma-aminobutyric acid (GABA) metabolism in synaptosomal preparations. In the present investigation, we have assessed the interaction between paraoxon and the GABA system at synaptic level.

Methods: Synaptosomes were prepared from male Wistar rats (200-250 g). Cerebral cortex was dissected and homogenized, then centrifuged at 1000g for 5 min and again at 12000g for 20 min. The pellet containing synaptosomes was resuspended in the buffer solution and the protein concentration adjusted at 1 mg/ml. Determination of lactate dehydrogenase (LDH) activity, as a biochemical index for synaptosomal integrity, was assessed. Cholinesterase activity of synaptosomes was also determined. Synaptosomes were preincubated for 20 min with two concentrations of paraoxon (10^{-9}-10^{-3}M), and then incubated with [^{3}H]GABA for 10 min, before being washed through 0.65 μm filters.

Results: Paraoxon inhibited cholinesterase activity of synaptosomes in a concentration dependent manner. Synaptosomal accumulation of [^{3}H] GABA/GABA was time dependent and peaked at 15 min. Paraoxon significantly increased the uptake in nano molar concentrations and decreased it at higher concentrations.

Conclusion: The result of this study indicate that in synaptosomes prepared from rat cerebral cortex paraoxon increases GABA uptake at low dose and inhibits its uptake at high doses. This may imply a role for organophosphate-induced convulsion, which needs further clarification.
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Keywords ● Synaptosome ● GABA ● organophosphate ● paraoxon

Departments of [1]Physiology
and [2]Neurosurgery,
School of Medicine,
[3]Research Center of Chemical Injuries,
Military Medicine Institute,
Baqiyatallah Medical Sciences University,
Tehran, Iran.

Correspondence:
Asghar Ghasemi MSc,
Department of Physiology,
School of Medicine,
Baqiyatallah Medical Sciences University,
Tehran, Iran.
Tel/Fax: +98 21 22281561
E-mail: as_qasemi@yahoo.com

Introduction

Organophosphates (OPs) are toxic substances used as pesticides, insecticides, and chemical warfare agents and their use has raisen thremnedously.[1,2] Because of its stability in aqueous solutions, parathion among OPs is regularly used as an insecticide and most likely is responsible for most accidental poisonings.[3,4]

The most striking clinical symptom in OPs poisoning is seizure or convulsion.[5] Neural injuries induced by OPs have strong associations with OP-induced seizures.[6] Although, the

main mechanism of OPs is cholinesterase inhibition leading to the accumulation of acetylcholine; their non-cholinergic effects are recently have attracted more attention.[7] Many investigators have indicated that, in addition to cholinergic system, other neurotransmitters may be involved in OP-induced convulsions.[7-11]

On the other hand, medical doctrine for nerve agent assault, including pyridostigmine pretreatment followed by anticholinergic and oxime therapy, does not ameliorate nerve agent-induced seizure activity.[5,8] It has been proposed that the increased seizure susceptibility may be due to instabilities in neuronal networks caused by excessive excitatory transmission and/or impaired inhibitory transmission mediated by the amino acid transmitters such as glutamate or gamma-amino butyric acid (GABA) respectively.[12] McDonough and colleagues have reported that changes in the GABA level during nerve agent convulsion are controversial and it is unlikely that brain GABA system cause OP-induced seizures.[5] It has been suggested that nerve agents may inhibit GABA metabolism in synaptosomal preparations.[5]

Paraoxon is the metabolic product of parathion which causes cholinesterase inhibition with low propensity to aging and good reactivatability.[13] Keeping in mind the above notion it is possible that organophosphates affect GABA turnover in the brain. Synaptosomes constitute a useful *in vitro* model to study neurotransmitter uptake and release because they retain many properties of nerve endings.[14] It is reported that synaptosomal GABA content may change differently from whole brain GABA content,[15] therefore, we have attempted to determine the effect of paraoxon on GABA uptake by the rat cortical synaptosomes.

Materials and Methods

Male Wistar rats (200-250 g) were kept in 22±2ºC and 12h/12h light dark cycle. Their access to food and water was *ad libitum*. All animal experiments were according to the established protocols by the Ethical Committee of the University.

Synaptosome preparation

Synaptosomes were prepared as previously described by Raiteri and colleagues.[16] In brief, rat cerebral cortex was dissected and homogenized in 0.32 M sucrose in 100 mM phosphate buffer, pH=7.40. The homogenate was then centrifuged at 1000g for five min in order to remove cell debris and then the supernatant was re-centrifuged at 12000g for another 20 min. The pellet, containing synaptosomes, was resuspended in buffer solution containing (in mM): [NaCl= 125, KCl= 3, $MgSO_4$= 1.2, $CaCl_{2=}$ 1.2, $NaHCO_3$= 22, NaH_2PO_4= 1] and pH= 7.4, oxygenated with 95% O_2 and 5% CO_2. Protein concentration was adjusted at 1 $mg.ml^{-1}$. In [^{3}H]GABA (Amersham; UK) uptake experiments synaptosomes prepared from three rats were used in triplicate for each paraoxon concentration. There was a control group in each run of experiments.

Protein concentration determined by Bradford method,[17] and bovine serum albumin (BSA, Fluka; Swiss) was used as standard. Determination of LDH activity, as a biochemical proof for synaptosomal integrity, was assessed with reduction of pyruvate to lactate.[18] Oxidation of NADH to NAD^+ was also monitored at 339 nm using Spectrophotometer (LKB Biochrom Novaspec, UK). Synaptosomal LDH activity was measured in the presence (total activity) and the absence (free activity) of 1% Triton X-100 and values expressed as percent of total. Cholinesterase activity was determined by Ellman method as previously described by Dietz.[19]

[^{3}H]GABA uptake assay

Synaptosomes (1mg protein/ml) aliquoted into 0.5 ml, then 10 µM Aminooxyacetic acid (AOAA, Sigma; Germany) was used to prevent GABA metabolism during experiment. Synaptosomes were preincubated for 20 min with paraoxon (10^{-9}-10^{-3}M), and incubated with 400 nM GABA (1% of which was tritiated) for 10 min at 37ºC subsequently reaction was stopped by 1 ml of cold saline. Synaptosomes were layered on 0.65 µm filters (Millipore), after three times washing, the filter radioactivity were counted with liquid scintillation counter. Specific GABA uptake was calculated as total uptake minus uptake in presence of 50 mM nipecotic acid (an inhibitor of GABA uptake). In time course studies uptake was measured after 2.5, 5, 7.5, 10, 15, 20, and 30 min exposure of synaptosomes to [^{3}H]GABA/GABA.

Data are expressed as mean±SEM. Comparison between groups was done by paired Students' *t* test or One-way ANOVA with Tukey's HSD test if necessary and $P<0.05$ was considered as statistically significant.

Results

LDH activity

When expressed as percent of total, occluded and free LDH activity were 87±1% and 13±1% respectively ($P<0.05$, n=8). Total LDH activity was 465±31 nmol/min.mg protein. In order to determine if high doses of paraoxon *per se* affects membrane integrity, we attempted to compare the occluded LDH activity before and after paraoxon exposure. Our results did not

demonstrate any significant variations (88% vs. 85%, n=3, duplicate, Fig. 1).

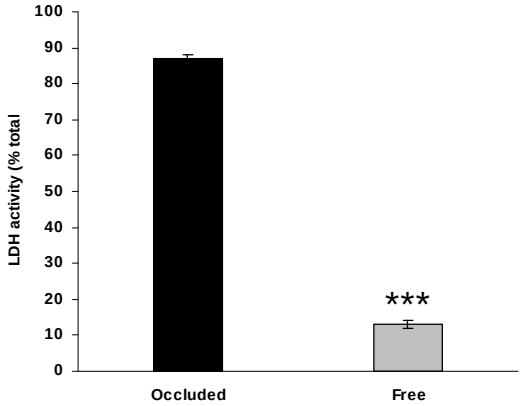

Fig 1: Occluded and free LDH activity (percent of total) of synaptosomes. Occluded LDH activity was significantly higher than free, which indicated synaptosomes had membrane integrity (P<0.001). Total LDH activity was 465±31 nmol/min.mg protein.

Cholinesterase activity

Paraoxon inhibited cholinesterase activity of synaptosomes, and this inhibition was concentration-dependent. 50% inhibitory concentration of Paraoxon (IC_{50}) for cholinesterase inhibition was approximately 10 nM (Fig. 2).

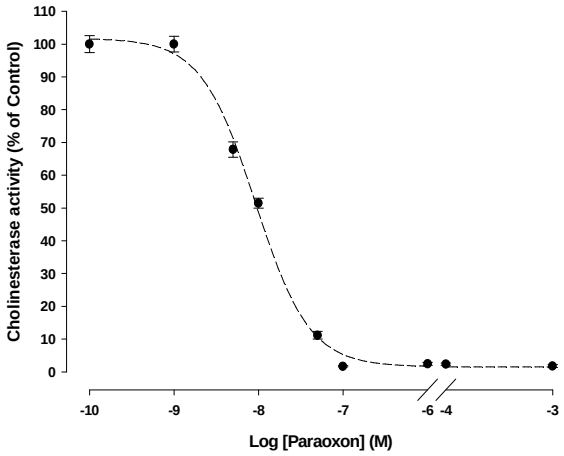

Fig 2: synaptosomal cholinesterase inhibition by paraoxon. IC50 for inhibition is approximately 10 nM. Cholinesterase activity in control group was 78±7 nmol/min. mg protein. ***: P<0.001 compare to control.

Time dependency of [^{3}H] GABA uptake

Synaptosomal accumulation of [^{3}H] GABA/ GABA was time dependent and peaked at 15 min. [^{3}H] GABA uptake were 22, 35, 49, 72, 101, 100, and 100 pmol/mg proteins at 2.5, 5, 7.5, 10, 15, 20, and 30 min passed the beginning of incubation respectively. The increase was not significant until 10 minutes through incubation time (Fig. 3).

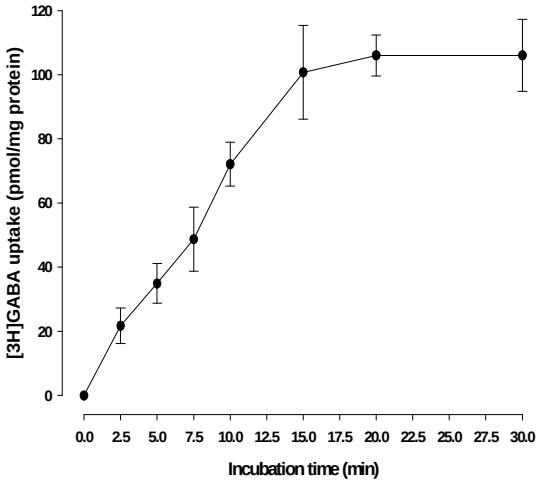

Fig 3: Time dependency of GABA uptake. Uptake reached a maximum at 15 min.

Effect of paraoxon on [^{3}H] GABA uptake

Paraoxon had a quasi-biphasic effect on [^{3}H] GABA uptake in rat cortical synaptosome. Paraoxon increased the uptake in nanomolar concentrations (10^{-9}-10^{-6}M) and decreased it in micromolar concentrations (10^{-5}-10^{-3}M). Increase in the uptake was significant at 10^{-8}-10^{-6}M (P<0.01), albeit an observable increment at greater concentrations. The situation reversed at 10^{-3} M paraoxon, where the decrease in uptake was statistically significant (P<0.05, Fig. 4).

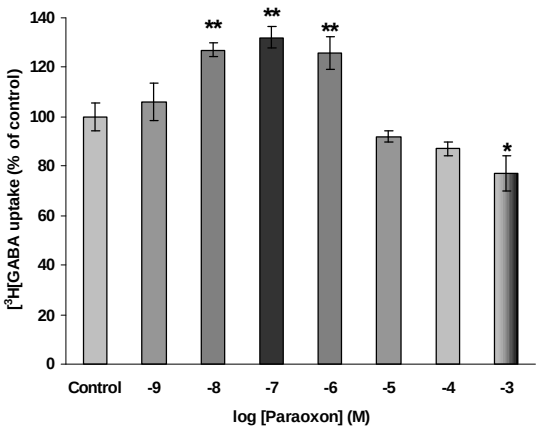

Fig 4: Effect of paraoxon on [^{3}H]GABA uptake by rat brain synaptosome. *: P<0.05, **: P<0.01, compare to control. [^{3}H]GABA uptake in control group was 55±3 pmol/mg protein.

Discussion

In this study we found that paraoxon had a dual effect on GABA uptake. It increased the uptake at nanomolar concentrations and decreased it at higher doses. Paraoxon did not invade membrane integrity, as confirmed by LDH experiments.

Similar values of LDH activity before and after paraoxon exposure indicated that paraoxon did not disrupt membrane integrity.[20] In addition, occluded LDH activity was 87% of total, a value in good accordance with other reports.[21,22] We observed that synaptosomal [^{3}H]GABA uptake had time dependency and reached to its maximum value at 15 min. This result in part is consistent with the reports of Sutch and colleagues demonstrated that [^{3}H]GABA uptake in the rat cortical synaptosome was maximum at 20 min.[23] Neal and Iversen (1969) suggested that isolated synaptosome accumulate [^{3}H]GABA in a time dependent manner.[24] Low doses of paraoxon increased GABA uptake while its high doses had an opposite effect. This finding is somewhat supported by the results of Ho et al. who found that toxic doses of diisopropylphosphofluoridate, an Ops, increased and then decreased GABA uptake after 6 and 24 hours respectively.[25] Other researchers working on soman, another Op, have reported that it either does not affect GABA levels in the rat brain,[26] or it even increases it significantly in guinea pig cerebral cortex.[27] Beckman *et al.* reported that muscarine (an agonist for muscarinic acetylcholine receptors) decreased [^{3}H] GABA uptake in culture of rat hippocampus.[28] Hence it is possible that high doses of paraoxon (possibly due to its cholinesterase inhibition or its direct effect) may activate muscarinic receptors resulting to a decrease in GABA uptake.

Direct interactions of OPs with muscarinic receptors have been observed at concentrations that inhibit cholinesterase or even at lower doses.[29] Lotti *et al*. explained that in most cases, *in vitro* OP concentrations used to affect receptors directly were higher than those inhibiting Acetylcholinesterase (AChE); therefore the toxicological significance of these direct interactions is not understood.[30] Szilagyi and coworkers showed that 1-2 mM tabun (an OP agent) decreased [^{3}H] GABA uptake in guinea pig cerebral cortex,[31] which supports the inhibitory effect of the high dose (1mM) of paraoxon in this study, and provide an evidence for involvement of GABA system in OP effects.

Changes in the level and the function of GABA during nerve agent seizures have been controversial. Reports have showed that brain GABA levels were increased,[5] decreased,[32] or did not change in rats following organophosphate intoxication.[33] A consequence of the observed increase in GABA uptake is that it could reduce the amount of GABA in the synaptic cleft. This will reduce post-synaptic $GABA_A$ receptor or presynaptic $GABA_B$ receptor activation; $GABA_A$ receptors mediate inhibitory actions on post-synaptic sites while $GABA_B$ receptors reduce the release of excitatory amino acid transmitters,[34] therefore, both may lead to excitation and probably to convulsion. Pharmacological blockade of GABA transporters by tiagabine has inhibited seizure.[35]

Increasing GABA uptake disturbs inhibition/excitation balance in benefit of excitation. This imbalance works in favor of hyperexcitability and may lead to convulsions. This is, at least in part, against previous believes that the brain GABA system is the implausible cause of OP-induced seizures.[5] Zhao *et al.* reported that GABA transporters might play an important role in epileptogenesis, and this may be related to alterations in balancing the excitatory and inhibitory synaptic interactions.[35] In present study paraoxon IC50 for cholinesterase inhibition was approximately 10 nM, although other IC50 values of 13.67 nM,[36] and 20 nM,[37] were reported in other studies.

Conclusion

The results of this study show that paraoxon at low dose increased and at high dose decreased [^{3}H] GABA uptake in synaptosomal preparations made from the rat cerebral cortex. This effect may suggest a role for GABA transporters in organophosphate-induced convulsion.

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