Involvement of the GABAergic System in Dimethoate-induced Intoxication

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The alteration of the GABAergic system in dimethoate intoxication was explored. A rat brain AChE (acetylcholinesterase) activity and GABAergic profiles, including the level of gamma-aminobutyric acid (GABA) and the densities and affinities of gamma-aminobutyric acid A ($GABA_A$) receptors in the hippocampus, were examined 2 hr after dimethoate administration (0, 38.9, 83.7 and 180.0 mg/kg bw po). The AChE activity was reduced by 60–70% by all three doses of dimethoate. Statistically significant elevation of GABA levels was observed following the administration of dimethoate 180.0 mg/kg bw (121.5% of control, $p < 0.05$), while the B_{max} values for the $GABA_A$ receptors of the hippocampal synaptic membranes were 50.6% and 51.6% of control values in the 83.7 and 180.0 mg/kg bw dimethoate groups, respectively ($p < 0.05$). Statistically significant decreases in the K_d values of 19.8, 51.7, and 53.4% *vs.* controls were observed in a dose-dependent manner ($p < 0.05$). In conclusion, it is suggested that the GABAergic system is maybe involved in the neurotoxicity of organophosphates as well as the cholinergic mechanisms.

Key words —— gamma-aminobutyric acid, noncholinergic mechanism, organophosphates

INTRODUCTION

Organophosphorus compounds (OPs), such as dimethoate, parathion, and omethoate, have long been major insecticides used for insect control in a variety of crops. The acute toxicity of OPs are believed to be due primarily to the inhibition of acetylcholinesterase (AChE) resulting in an accumulation of acetylcholine (Ach) with a sustained overstimulation of Ach receptors in the clefts of central and peripheral neuronal synapses.[1)] They can cause a progression of toxic signs, including hypersecretion, convulsions, respiratory distress, coma, and death. Most of the health risk assessments of OPs are mainly based on the involvement of the cholinergic mechanism in the OP intoxication such as the current treatment regimen with atropine and oximes.[2)] However, this treatment strategy seems to be far from the optimal approach, particularly due to its serious side effects at high dosages of atropine and the limited potential of the oximes.[2,3)]

Therefore numerous studies have been carried out to elucidate the underlying mechanisms not only for cholinergic but also noncholinergic damage with particular attention to the recruitment of the excitatory amino acids (EAAs) in intoxication during the past decades.[4–7)] Further more, alternative treatments based on the EAA system have also been assessed in an attempt to control the seizures and achieve more survivors after severe acute OP poisoning.[2,7–9)]

The inhibitory amino acid (IAA) system, which has important physiologic and pathologic roles in anesthesia and epilepsy, has been neglected in the exploration of mechanisms for OP intoxication. However, evidence for neuronal coexistence of the GABAergic system including gamma-aminobutyric acid (GABA), gamma-aminobutyric acid A ($GABA_A$) receptors, and its uptake system in some cholinergic neurons have been demonstrated in previous anatomic investigations.[10–17)] Furthermore, other studies have demonstrated the interactions between cholinergic and GABAergic activities in the thalamus, neostriatum, striatum, and hippocampus involved in both physiologic and patho-

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logic conditions.[18–22] It was demonstrated that dopaminergic, GABAergic, and cholinergic interactions regulate the concentration of Ach in the stratium.[23] Dewey *et al.* found that drugs acting upon GABAergic neurons produce profound regional changes in Ach release.[18] In contrast, the release of GABA from the thalamus is inhibited by Ach through the activation of M2 receptors.[21] In thalamus sensory nuclei, nicotinic Ach receptor activation facilitated the release of the inhibitory neurotransmitter GABA.[24]

Since the confirmation of the structural coexistence and functional interactions between the cholinergic and the GABAergic activities, more approaches are needed to understand the involvement of the GABAergic mechanisms after irreversible cholinesterase inhibition in OP intoxication. In this study, brain AChE activity and GABAergic profiles including GABA neurotransmitters and the $GABA_A$ receptor were examined in the rat hippocampus after 2-hr of dimethoate administration to explore the role of the IAA system in OP poisoning.

MATERIALS AND METHODS

Chemicals —— Dimethoate, 99% pure in crystalline form, was a gracious gift from Yunfa Chemical Co., Ltd. (Shanghai, China); GABA, DL-homoserine, thioacetyl-choline iodide (ASCh), 5,5-dithio-bis-nitrobenzoic acid (DTNB), physostigmine, and reduced glutathione were purchased from Sigma Chemical Co.; *O*-phthaladehyde (OPA) and β-mercaptoethanol (2-MCE) were from Fluka Chemical Co.; and [^{3}H]GABA (35 Ci/mmol) was obtained from China Isotope Corporation (Beijing, China). All other chemicals were verified to be analytically pure reagents.

Preparation of Solutions —— Dimethoate was prepared in corn oil. Ice-cold artificial cerebrospinal fluid (ACSF) contained NaCl 130 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgSO_4$ 2 mM, NaH_2PO_4 1.25 mM, $NaHCO_3$ 26 mM, glucose 10 mM, and sucrose 10 mM, pH 7.4. For the AChE activity assay, ASCh and physostigmine were dissolved in saline. DTNB was dissolved in buffered phosphate (0.1 M, pH 8.0) as stock solution and diluted with saline at use. Reduced glutathione was prepared in distilled water. The stock solutions of GABA and DL-homoserine as internal standards were prepared at the concentration of 1 mM in filtered K_2CO_3 solution (0.1 M) containing 50% HPLC grade methanol and stored at −20°C for GABA determination. In the stock derivatizing solution, 13.4 mg of OPA was dissolved in 1 ml of dehydrated alcohol and 20 ml of 2-MCE was then added. The solution was made with diluted in 4 ml of sodium tetraborate solution (0.1 M, pH 9.6) and stored protected from light at 4°C. The working derivatizing solution was made daily by adding 20 µl of the 2-MCE to maintain the hydrosulfide concentration. All of the working solutions were freshly diluted from stock solutions.

Experimental Animals and Treatment —— Twenty-four male Sprague-Dawley rats (200 ± 20 g) were obtained from the Animal Research Center of Fudan University. The animals were conditioned in a temperature-controlled environment with a 12/12-hr light/dark cycle and allowed free access to water and food for 1 week before the experiment started. All experimental procedures were carried out in accordance with the principles stated in the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (U.S.A.) as well as Fudan University. The rats were randomly divided into four groups that received 0, 38.9, 83.7, and 180.0 mg/kg bw of dimethoate via oral gavage. Corn oil served as the vehicle control. The administered volume of each dose was 10 ml/kg bw.

Observation of Animals —— All animals were observed for overt neurobehavioral function status before and after drug administration. Specific signs related to OP poisoning, such as salivation, fasciculations, tremors, facial movements, head clonus, forelimb clonus, chewing, and Straub tail were extensively observed and recorded. All animal experimental procedures were performed between 9:00 and 14:00.

Neurochemical Determinations —— Following decapitation, the brains were quickly removed from rats and the hippocampi were dissected above an ice box as quickly as possible. The samples were frozen in liquid nitrogen and stored at −80°C until analysis. The hippocampi were homogenized with 8–10 strokes using a glass homogenizer in ice-cold ASCF (1 : 9, w/v). The homogenates were collected and centrifuged at 4°C for 15 min at 3000 rpm for subsequent AChE activity, GABA concentrations and total protein assays. For the [^{3}H]-GABA binding assays, the hippocampi were homogenized in ice-cold sucrose solution (0.32 M, pH 7.4, 1 : 9, w/v) for synaptic membrane preparation. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant was removed and

centrifuged for 20 min at 20000 × *g*. The pellet was resuspended in Tris-HCI buffer 50 mM (pH 7.4) and centrifuged at 48000 × *g* for 30 min. The last step was repeated. The resulting pellet was frozen and stored at −70°C for at least 18 hr before use. On the experiment day, membrane pellets were thawed at 4°C and resuspended in 2 to 3 volumes (v/v) of Tris-HCI buffer 50 mM containing 0.05% nonionic detergent Trition X-100 before centrifugation at 48000 × *g* for 30 min. The final pellet was then resuspended in Tris-HCI buffer 50 mM to a final concentration of 200 μg/ml to 350 μg/ml and used for binding assays.

Hippocampal AChE Activity Assay —— Total AChE activity was determined using Ellman's colorimetric method.[25] Briefly, a 30 μl of aliquot of homogenate was added to 3 ml of phosphate buffer containing DTNB and ASCh and then activity was determined using a visible range spectrophotometer (Unico, Shanghai, China) at a wavelength of 412 nm. The AChE activity in hippocampus was calculated as μmoles of ASCh hydrolyzed per gram of protein per minute (μmole/g·min).

Determination of Hippocampal GABA Levels —— After homogenization and centrifugation, the amino acids were derivatized in the working derivatizing solution of OPA and 2-MCE. Then the GABA concentration was determined using a HP-1100 HPLC system and a fluorescence detector (Hewlett-Packard, Germany) by isocratic elution as previously described.[26] A Hypersil ODS-3 column (4.6 × 250 mm, 5 μm, GL, Japan) was used. The mobile phase was potassium dihydrogen phosphate buffer 0.1 M (pH 6.0) containing 30% methanol and 10% acetonitrile delivered at a flow rate of 1.0 ml/min which was filtered through a 0.2-μm nylon membrane and degassed by ultrasonification before use. DL-homoserine was served as the internal standard for quantification.

[^{3}H]-GABA Binding Assays —— The binding assays were performed in duplicate in a final volume of 300 μl of buffer at room temperature for 45 min as described by Enna and Synder with minor modifications of six increasing concentrations of [^{3}H]-GABA ranging from 0.05 nM to 60 nM.[27] Nonspecific binding was determined in the presence of 5 mM of unlabeled GABA. The incubation was terminated by rapid filtration through glass fiber filters pretreated with 0.15% polyethyleneimine for at least 2 hr to reduce filter binding. The radioactivity retained on the filters was counted with liquid scintillation spectrometry.

Determination of Protein —— The supernatant was collected after centrifugation at 3000 rpm and the total protein concentration was measured using the bicinchoninic acid method kit according to the instructions of the manufacturer (Beyotime, China) to correct the total AChE activity and GABA concentration.

Data Analysis —— AChE activity, GABA concentration, and densities and affinities of $GABA_A$ receptors are expressed as percentages of control values. Statistical comparisons among different groups were made using one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* tests. For the binding assays, the B_{max} and K_d values of $GABA_A$ receptor were determined by computerized Scatchard analysis with nonlinear regression (GraphPad Prism Program, version 4.0, San Diego, CA, U.S.A.). All other statistical analyses were performed using SPSS (version 11.5) software and statistical significance was represented by p values of less than 0.05.

RESULTS

Dimethoate-induced Toxicity and Brain AChE Activity

The animals treated with dimethoate showed typical signs of OP-induced toxicity, which included weakness, salivation, fasciculations, tremor, facial movements, and head and forelimb clonus. Severe manifestations, such as unconsciousness, incontinence, and convulsions, were noted in the dimethoate group 180.0 mg/kg bw. There was no death during the course of brain AChE and GABA assays. Significant inhibition of AChE activity in the rat hippocampus was observed at all doses of dimethoate in a dose-dependent manner (Fig. 1). The percentages of brain AChE activity in the 38.9, 83.7, and 180 mg/kg bw groups were 42.1% ($p < 0.05$), 35.7% ($p < 0.05$), and 30.1% ($p < 0.05$) as compared with controls, respectively.

GABA Concentrations in Rat Hippocampus Following Dimethoate Administration

The levels of GABA showed a slight increase in the rat hippocampus in the 38.9 mg/kg bw (113.5% of control) and 83.7 mg/kg bw dimethoate (115.6% of control) groups 2 hr following administration ($p > 0.05$). This increase reached significant levels in animals treated with dimethoate 180.0 mg/kg bw (121.5% of control, $p < 0.05$; Fig. 2). This im-

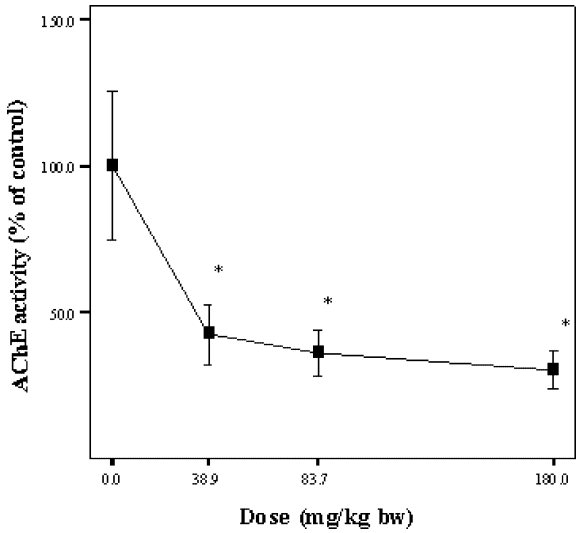

Fig. 1. Hippocampal AChE Activity at 2 hr After Dimethoate Administration.

Rats were Administrated with Dimethoate (0, 38.9, 83.7 and 180.0 mg/kg bw) Dissolved in Corn Oil via Gavage. Data were presented as percentage of controls and mean ± S.D. were given (n = 6) for each group. $^{*}p < 0.05$ compared to the control group.

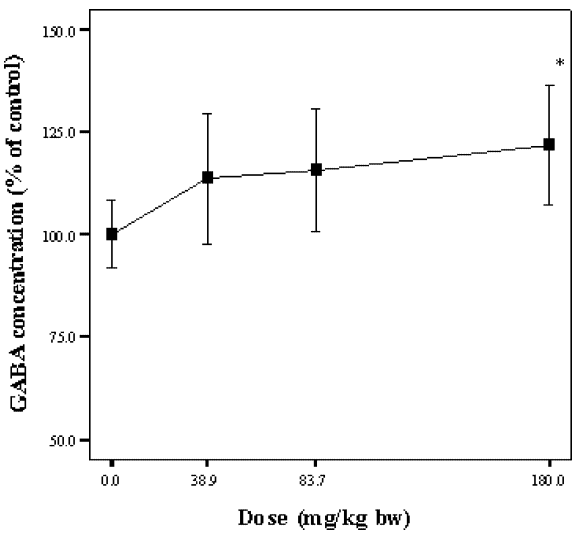

Fig. 2. GABA Concentrations in Hippocampus from Rats at 2 hr After Dimethoate Administration.

Rats were Administrated with Dimethoate (0, 38.9, 83.7 and 180.0 mg/kg bw) via Gavage. Data were presented as percentage of controls and mean ± S.D. were given (n = 6) for each group. $^{*}p < 0.05$ compared to the control group.

plies that GABA metabolism might be involved in dimethoate-induced cholinergic processes in the rat hippocampus.

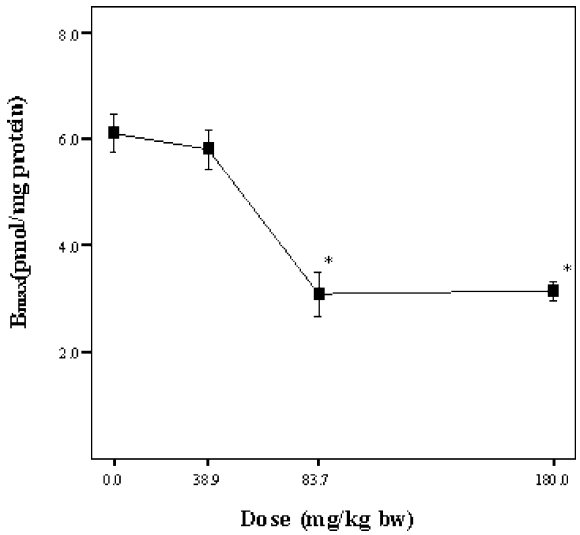

Fig. 3. The B_{max} values of [^{3}H]-GABA Binding to Hippocampal Synaptic Membranes from Rats Treated with Dimethoate (0, 38.9, 83.7 and 180.0 mg/kg bw) 2 hr.

Membranes were incubated with ligand concentrations ranging from 0.05 nM to 600 nM. Values indicated represent mean ± S.D. of three independent experiments. Values significantly different from controls, by one-way ANOVA and Dunett's *post-hot* tests ($^{*}p < 0.05$).

Densities and Affinities of $GABA_A$ Receptors in the Rat Hippocampus Following Dimethoate Administration

The maximum density of binding sites ($B_{\max}$) for [^{3}H]-GABA to the hippocampal synaptic membranes of rats is shown in Fig. 3. The $B_{\max}$ value in the 38.9 mg/kg bw group represented a mild decrease (94.8% of control) compared with the control ($p > 0.05$). However, the numbers of binding sites were 50.6% and 51.6% of control in the dimethoate 83.7 mg/kg bw and 180.0 mg/kg bw groups, respectively. Both were statistically different from that of controls ($p < 0.05$).

The effect of dimethoate administration on $GABA_A$ receptor binding affinity was also been determined. Two hours following dimethoate administration, statistically significant decreases in the K_d values were observed at all doses of dimethoate with a dose-dependent tendency in the hippocampal membranes, as shown in Fig. 4. The percentages of the K_d values were reduced by 19.8, 51.7, and 53.4% of controls in the dimethoate 38.9, 83.7, and 180.0 mg/kg bw groups, respectively. A typical saturation binding curve with an inset Scatchard plot is shown in Fig. 5.

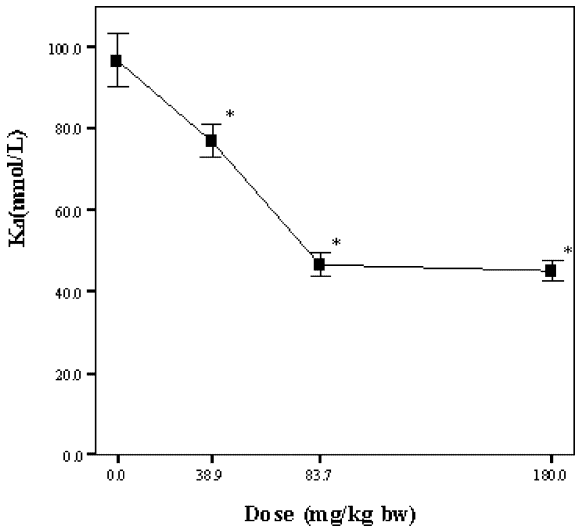

Fig. 4. The K_d Values of [^{3}H]-GABA Binding to Hippocampal Synaptic Membranes from Rats Treated with Dimethoate (0, 38.9, 83.7 and 180.0 mg/kg bw) 2 hr.

Membranes were incubated with ligand concentrations ranging from 0.05 nM to 600 nM. Values indicated represent mean ± S.D. of three independent experiments. Values significantly different from controls, by one-way ANOVA and Dunett's *post-hot* tests ($^*p < 0.05$).

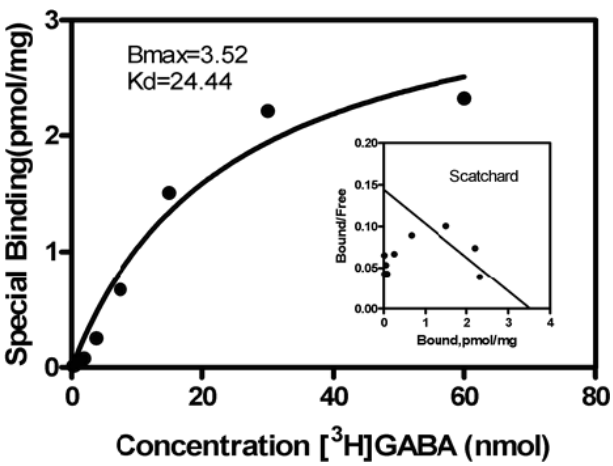

Fig. 5. Representative Saturation Curve of Specific [^{3}H]-GABA Binding to Synaptic Membranes from a Dimethoate (83.7 mg/kg) Treated Adult Rat Brain Hippocampus.

Membranes were incubated in triplicate with ligand concentrations ranging from 0.05 nM to 60 nM. The inset shows the respective Scatchard transformation plot.

DISCUSSION

Disorders of the cholinergic mechanism play a vital role in OP-induced intoxication. However, some neurotoxicity of OPs cannot be explained solely by their direct actions on AChE. The relationships between the cholinergic and amino acid neurotransmitter systems, especially the EAAs, have been implied in some experimental studies.[6, 28] But the role of the IAAs has been little known in OP intoxication. In the present study, we examined the alterations in GABA levels and $GABA_A$ receptors characteristics in dimethoate-induced poisoning to elucidate the noncholinergic mechanisms involved in OP intoxication.

The GABA level increased to 113.5–121.5% of the control level in the rat hippocampus 2 hr after dimethoate administration. This correlates well with other observations. Shih and McDonough reported that GABA concentrations reached significantly high levels in the hippocampus, cortex, and striatum after seizure onset following soman intoxication.[4] Similarly, a marked increase in extracellular striatal levels of GABA was observed in rats with severe signs of poisoning after soman administration.[29]

In addition, changes in the densities and function of the GABA receptors have also been investigated following nerve agent exposure. The acute administration of diisopropylphosphorofluoridate increased the number of GABA receptors without affecting the muscarinic receptor characteristics in rat brain striatum at 6 hr.[30] But the results from the present study demonstrated that a decreased density of $GABA_A$ receptors in rat hippocampal synaptic membrane 2 hr following dimethoate administration. Furthermore, after acute administration of dimethoate (38.9, 83.7, and 180 mg/kg bw) the K_d values of hippocampal $GABA_A$ receptors were decreased by 19.8, 51.7, and 53.4% of control values, respectively. Hence, although the densities of $GABA_A$ receptors in the hippocampus were reduced, the affinities of the receptors were enhanced in dimethoate intoxication in rats. The apparent discrepancy of these $GABA_A$ receptor density findings with those of Sivam *et al.* may be due to the differences between the two OPs, dimethoate and diisopropylphosphorofluoridate, and may be related to the different determination time after administration. This requires further study.

It is hypothesized that the initiation and expression of seizures are cholinergic phenomena according to a three-phase model of the neuropharmacologic processes responsible for the seizures and neuropathology induced by AChE inhibitors.[31] Conceptually, the progression of events following OP poisoning can be divided into the following three phases: an early cholinergic phase with the cholinergic hyperstimulation; a transitional phase

of progressively mixed cholinergic or noncholinergic modulation; and finally a predominantly noncholinergic phase. In the current study, the recruitment of the GABAergic system including elevated GABA levels and the affinities of $GABA_A$ receptors probably occurred in the second phase and the increased inhibitory response may be an attempt to overcome the heightened neuronal excitation 2 hr after dimethoate administration. In conclusion, the present data demonstrate that the GABAergic system is involved in the neurotoxicity of OPs as well as cholinergic mechanisms.

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