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Effects of Intracerebroventricular Administration of Ethylcholine Aziridinium (AF64A) on Dopaminergic Nervous Systems

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Changes in dopaminergic activities were investigaed after the intracerebroventricular (icv) administration of ethylcholine aziridium (AF64A) in rats. The levels of dopamine (DA) and metabolites, the activities of tyrosine hydroxylase (TH) and monoamine oxidase (MAO), and the specific binding sites of dopamine receptors in striata, hippocampus, and frontal cortex were assessed 6 days after the AF64A treatment with 3 nmol/each ventrcle. In frontal cortex, the levels of DA and metabolites were significantly decreased without changes in metabolites/DA ratios in the AF64A-treated groups. In contrast, the ratios of metabolites/DA were significantly decreased in striatum and hippocampus in the AF64A treatment. The activity of TH in frontal cortex was significantly decreased. However, that in other areas was not changed. Also the activity of MAO-A was not changed in the studied brain regions. However, the activity of MAO-B in striatum was significantly increased with no change in other areas. The specific binding sites of dopamine D1 and D2 receptors were increased in AF64A-treated frontal cortex. However, those were not changed in striatum and hippocampus except the small decreased specific binding sites of dopamine D-1 receptors in striatum after AF64A treatment. These results indicate that the dopaminergic activity was altered in AF64A treatment. Furthermore, it suggests that the decreased dopaminergic activities in each brain regions might be differently affected by AF64A treatment.

Key words : Ethylcholine aziridinium, Dopamine metabolism, Tysosine hydroxylase, Monoamine oxidase, Dopamine receptors

INTRODUCTION

In Alzheimer disease (AD), the disturbances of the picture are dominant in the early symptoms. Also the inteligence impairments, especially memory disturbances, is known as the main symptom. Although the causes of the disease are still controversial, it is known that the central cholinergic nervous systems are closely related to the learning impairment. However it has been reported that the activities of other central nervous systems, such as dopaminergic nervous system, are also changed in this disease state.

It has been reported that monoamine neurons in aging and Alzheimer's disease are altered (Palmer and Dekosky, 1993). It has been reported that dopamine agonist and antagonist affect learning abilities in animals (Ichihara *et al.*, 1989; Levin *et al.*, 1989; Roberts *et al.*, 1994). A number of evidences accumulated that cholinergic as well as dopaminergic nervous systems might be involved in the learning behaviors.

Ethylcholine aziridinium ion (AF64A) is a nitrogen mustard analog of choline which is selective, irreversible neurotoxin. The AF64A-treated animals have been reported to lose the memory (Chrobak *et al.*, 1988; Nakahara *et al.*, 1988) and use for the animal models of Alzheimer' disease (Smith, 1988). Recently Lim *et al.* (1995) reported that AF64A impaired the motivation as well as the latent memory in Morris water maze.

It has been reported that AF64A induce the decreases in choline uptake, the destruction of the presynaptic terminals in cholinergic nervous systems (Bartus *et al.*, 1982; Leventer *et al.*, 1987; Ransmayr *et al.*, 1992) and the enlargement of ventrcle (Chrobak *et al.*, 1989). Although it has been reported that AF64A induced the changes in striatal dopamine and its metabolites level (Hortnagl *et al.*, 1987; Meana *et al.*, 1992), few investigated the changes in the other parameters of dopaminegic nervous activities after AF64A treatment. To improve the usefulness of

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AF64A for the amnestic animal models, it is necessary to investigate the various parameters of dopaminergic neuronal activities in different regions.

Therefore this study is designed to determine whether AF64A induces the changes in the various activities of the central dopaminergic neuronal system.

MATERIALS AND METHODS

Animals and materials

Four male Sprague-Dawley rats weighing 200-250 g were housed per cage at 22±2°C on a 12 h light/12 h dark schedule (8:00 a.m.-8:00 p.m.). Animals were freely accessible on food and water.

Acetylethylcholine mustard-HCl was purchased from Research Biochemical Inc. (Wayland, MA). Ethylcholine aziridinium (AF64A) is synthesized according to the method of Mantione *et al.* (1983). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Animal treatment

For the administration of AF64A, all surgical procedures were performed on male Sprague-Dawley rats with initial weights 250-300 g. Rats were anesthetized with Equithsin and mounted in a David Kopf stereotaxic apparatus. The skull was exposed and a guide cannula was implanted according to Paxino and Watson (1986) through the dural surface into the lateral ventricle with respect to bregma at the following coordinates: A -0.8, L +1.4, V ±4.4. Skull screws and dental cement were used for fixation of guide cannula. A stainless steel obturator was inserted into the guide cannula. Penicilline 30,000 I.U. was administered to protect from infection after surgery. Before the injection of AF64A, the rats were allowed to recover from surgery for 4 days, housed singly in their cages. AF64A was infused in both ventricle with the rate of 0.5 μl/min (3 nmol/each side) at four days after the surgery. The control groups were infused with the artificial cerebrospinal fluid. At six days after the infusion of AF64A, the rats were sacrificed.

Determination of DA and its metabolites

Rats were sacrificed by decapitation and striatum, hippocampus and frontal cortex were dissected out rapidly according to the method of Glowinski and Iverson (1966). The tissue samples were homogenated in 1 ml of ice-cold 0.05 μM perchloric acid (PCA). Following centrifugation (15,000 g for 20 min at 4°C), the supernatant was diluted with 0.05 M PCA for injection into the HPLC. The concentrations of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined according to the method described by Mayer and Shoup (1983) with a minor modification. Separations were achieved using a C18 reverse phase analytical column (5 μm spheres, 100×4.6 mm, Biophase ODS, Bioanalytical Systems, Inc.) and a mobile phase flow rate was 0.8 ml/min. The LC column was coupled to an electrochemical detector (M460, Waters Systems) equipped with a glassy carbon electrode set at a potential of 700 mV vs Ag/AgCl-3M NaCl reference electrode. The mobile phase was 10% acetonitrile/monochloroacetate buffer, pH 3.0 with 0.7 mM EDTA and 0.86 mM sodium octyl sulfate. The concentrations of dopamine and its metabolites were determined by direct comparison of sample peak heights to those of an external standard containing three neurochemicals.

Determination of tyrosine hydroxylase (TH) activity

TH activity in the dissected tissue was determined using the method described by Horwitz and Perlman (1984) with a minor modification. The accumulation of DOPA was measured in the presence of brocresine, an inhibitor of DOPA decarboxylase (Erny *et al.*, 1981). The tissue samples were homogenized in 1 ml of an ice-cold solution containing 10 mM sodium phosphate, 5 mM sodium pyrophosphate, 5 mM EDTA, and 0.2% Triton X-100, pH 6.7. One hundred microliter of homogenate was taken from each sample for protein content. The remaining homogenate was centrifuged at 15,000 g for 10 min at 4°C. One hundred microliter of supernatant was added to the 100 μl reaction solution composed of 80 μM L-tyrosine, 150 μM brocresine, 0.2 mM DL-6-methyl-5,6,7,8, -tetrahydropterin, 25×10^6 units of catalase, 100 μM $FeSO_4$, 100 mM 2-mercaptoethanol, and 100 mM 2-[N-morpholino]-ethanesulfonic acid (pH 6.8). Reactions were carried out for 10 min at 37°C and were terminated by the addition of 300 μl of 0.28 M trichloroacetic acid containing 90 pmol of epinephrine. After centrifugation at 15,000 g for 10 min, the supernatant was added to 20 mg of acid-washed alumina and pH was adjusted to 8.6-8.7 with 3 M Tris buffer containing 10 mM EDTA and 0.1 mM Na_2SO_3. The alumina was washed out four times with water, then DOPA was eluted with 300 μl of 0.1 M PCA. The eluent was assayed using HPLC-EC as described above except that acetonitrile was deleted in the mobile solution and the flow rate was 1.2 ml/min. TH activity is expressed as pmol DOPA produced/protein/min.

Determination of monoamine oxidase (MAO) activity

MAO activity was assayed by a minor modification of the method of Suzuki *et al.* (1979). The tissue samples were homogenated 1.8 ml of 0.1 M phosphate buffer. One hundred microliter of homogenate was

taken from each sample for protein content. The remaining homogenate was used as an enzyme source. The assay mixture consisted of 0.1 M sodium phosphate buffer (pH 7.4), enzyme solution (0.08 to 1.41 mg of protein), peroxidase solution (0.2 mg), homovanillic acid solution (0.5 mg), serotonin and benzylamine as a substrate of MAO-A and -B (10 μM), respectively, and water, 0.5 ml of each, to give a final volume of 3.0 ml. After incubation at 37°C for 60 min, the enzyme reaction was terminated by adding 0.1 ml of pargyline solution (0.2 mg), and the mixture was centrifuged at 18,000 g for 20 min at 4°C. The supernatant fraction was subjected to fluorescence measurement with exitation at 315 nm and with emission at 425 nm. Blank assays differed from controls only in that the substrate solution was added with pargyline after incubation. Standards were taken by adding 0.5 ml of hydrogen peroxide solution (4.42 to 22.1 nmoles) to the assay mixture instead of the enzyme solution. MAO activity is expressed as H_2O_2 nmol/hr/mg protein.

Membrane preparation for binding assays

Membranes for D_1 receptor binding assays were prepared according to the method of Zukin *et al.* (1974) with minor modifications. Briefly, the pooled samples were homogenized in 15 volumes of ice-cold 0.32 M sucrose using a Brinkmann Polytron PT-10 at a low speed. The homogenate was centrifuged at 1,000 g for 10 min, then the pellet was discarded and the supernatant was centrifuged at 20,000 g for 20 min. This crude synaptozomal pellet was resuspended in double distilled deionized water and dispersed with the polytron for 30 sec. The suspension was centrifuged at 8,000 g for 20 min. The supernatant, including the buffy layer was collected and was resuspended in water and centrifuged at 48,000 g for 20 min. The final pellet was suspended in 50 mM Tris-HCl buffer (pH 7.4) and stored at -70°C. Because much more tissue is required for D_2 receptor binding assy, membranes for D_2 receptor binding assay were prepared according to Carboni *et al.* (1985) with slight modifications. In brief, brain tissue was homogenated in 50 volume ice-cold 50 mM Tris-HCl buffer (pH 7.7) with an Ultraturex homogenizer. The resulting homogenate was centrifuged twice at 48,000 g for 20 min and the final pellet was resuspended in 50 mM Tris-HCl buffer containing 0.01% (w/v) ascorbic acid and the following ions: 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.4) and stored at -70°C.

Determination of [^{3}H]SCH-23390 binding sites

Specific binding sites of the D1 dopamine receptor ligand, [^{3}H]SCH-23390, was determined following the method of Porceddu *et al.* (1986) with a minor modification. The frozen membrane preparation was thawed and centrifuged at 48,000 g for 20 min. The pellet was suspended in 50 mM Tris-HCl (pH 7.4), containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$. Aliquots (200 μl) of the membrane preparations (0.3-0.5 mg protein/ml) were incubated with 0.4 nM of [^{3}H]SCH-23390 in a final volume of 1 ml. Nonspecific binding was determined by adding 1 μM unlabeled SCH-23390. After 30 min incubation at 37°C, the reaction was terminated by rapid filtration through Whatman GF/B glass fiber filters using a cell harvester. The filters were washed twice with 5 ml of ice cold buffer and then were transfered to scintillation counting vials contaning 10 ml of Safety Solve and counted.

Determination of [^{3}H]sulpiride binding sites

Specific binding sites of the D_2 dopamine receptor antagonist, [^{3}H]sulpiride, was determined following the method of Carboni *et al.* (1985) with a minor modification. The frozen membranes were thawed and centrifuged at 48,000 g for 20 min to obtain a pellet. The pellet was suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.01% (w/v) ascorbic acid and following salts: 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. Aliquots of 0.2 ml of the membrane preparation (0.5-1 mg protein/ml) were incubated with 2 nM of [^{3}H]sulpiride in a final volume of 1 ml. Nonspecific binding was determined by adding 1 μM unlabeled (-)sulpiride. Incubation were carried out for 30 min at 8°C. At the end of incubation, the samples were filtered under reduced pressure through glass fiber filter (GF/B, Whatman) using a cell harvester. The filters were then washed twice with 5 ml of Tris-HCl buffer and, after drying, were placed into scintillation vials with 10 ml of Safety Solve. Radioactivity retained in the filter was counted by liquid scintillation spectrometry. To reduce the binding of free [^{3}H]sulpiride, the filters were presoaked in 0.1% polyethylenimine solution.

Determination of protein concentration

The protein content of tissue homogenates was determined by the method of Lowry *et al.* (1951) using bovine serum albumine as a standard.

Statistics

The statistical significance of differences were determined using Student's t-tests.

RESULTS

Table I shows the level of DA and metabolites in

Table I. Effects of i.c.v. administration of AF64A on the level of dopamine and dopamine metabolites in the various rat brain regions

		DOPAC	DA	HVA
Striatum	Control	77.22±4.15	510.71±7.34	23.12±0.80
	Treated	71.49±2.34	519.36±14.18	23.04±0.59
Hippcampus	Control	0.72±0.03	1.13±0.06	-
	Treated	0.72±0.06	1.58±0.18**	-
Frontal Cortex	Control	10.23±0.32	51.39±2.55	3.86±0.06
	Treated	7.20±0.20**	36.62±22.69**	3.21±0.08*

Rats were sacrificed 6 days after the administration of AF64A and each regions were dissected out. The units were pmol/mg protein. The values are mean S.E.M.of four to six determinations. * P<0.05, ** P<0.01 compared to corresponding control values

Table II. Effects of i.c.v. administration of AF64A on the ratio of DOPAC/DA and HVA/DA in the various rat brain regions

		DOPAC/DA	HVA/DA
Striatum	Control	0.161±0.008	0.050±0.003
	Treated	0.138±0.003*	0.045±0.002
Hippcampus	Control	0.653±0.028	-
	Treated	0.458±0.014*	-
Frontal Cortex	Control	0.200±0.011	0.072±0.006
	Treated	0.199±0.013	0.089±0.008

Rats were sacrificed 6 days after the administration of AF64A and each regions were dissected out. The values are mean S. E.M. of four to six determinations. *P<0.05 compared to corresponding control values

Table III. Effects of i.c.v. administration of AF64A on the activities of tyrosine hydrosylase (TH) and monoamine oxidase (MAO) in the various rat brain regions

		TH	MAO-A	MAO-B
Striatum	Control	23.64±0.41	1.42±0.03	0.78±0.05
	Treated	25.02±0.82	1.46±0.08	0.97±0.02*
Hippcampus	Control	1.78±0.10	-	-
	Treated	1.63±0.28	-	-
Frontal Cortex	Control	1.85±0.07	0.17±0.03	0.23±0.03
	Treated	1.53±0.12*	0.15±0.02	0.24±0.03

Rats were sacrificed 6 days after the administration of AF64A and each regions were dissected out. The units of activities of TH and MAO were pmol DOPA produced/min/mg protein and H_2O_2 nmol/hr/mg protein, respectively. The values are mean±SEM of four to five seperate determinations. *P<0.05 compared to corresponding control values

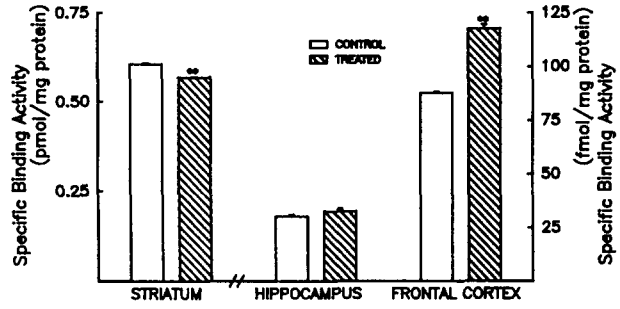

Fig. 1. Effects of icv administration of AF64A on [^{3}H]SCH 23390 binding activities in rat brain. Rats were sacrificed 6 days after the infusion of AF64A (3 mol/ each ventricle) and each brain regions were dissected out. Values are mean±S. E.M. for 4 seperate determination, done in duplicate. **P<0. 01 compared to corresponding control values

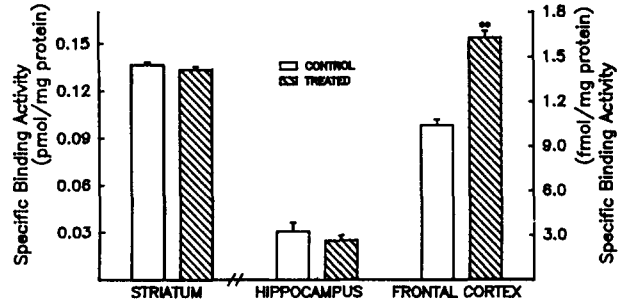

Fig. 2. Effects of icv administration of AF64A on [^{3}H]sulpiride binding activities in rat brain. Rats were sacrificed 6 days after the infusion of AF64A (3 mol/ each ventricle) and each brain regions were dissected out. Values are mean±S. E.M. for 4 seperate determination, done in duplicate. **P<0. 01 compared to corresponding control values

various brain regions at 6 days after the icv infusion of AF64A. The concentrations of DA and metabolites were not changed in the striatum. In hippocampus, the levels of DA was significantly increased (40%) without the changes in DA metabolites after AF64A-treatment. However the concentrations of both DA and metabolites were significantly decreased (38-17%) in frontal cortex of AF64A-treated group. Table II shows the ratios of DA to its metabolites in various brain regions after the icv infusion of AF64A. The ratios of DOPAC/DA in the striata and hippocampus of AF64A-treated group was significantly decreased without the changes in the ratio of HVA/DA. The decreased rates of DOPAC/DA in striatum and hippocampus were 14.3 and 30%, respectively. However the ratios of either DOPAC/DA or HVA/DA in frontal cortex was not changed.

The changes in the activities of TH and MAO in various brain regions after AF64A infusion was summarized in Table III. The activity of TH was significantly decreased (17%) in frontal cortex of AF64A-treated group. But the activity was not changed in

either striatum or hippocampus of the treated group. Also the activities of MAO-A were not changed in the examined regions of AF64A-treated group. However the activity of MAO-B was significantly increased (24%) in the striata of AF64A-treated group.

Fig. 1 and 2 shows the changes in the D-1 and D-2 specific binding sites after the infusion of AF64A. D-1 specific binding sites in the striata of AF64A-treated group was small but significant decreased. However D-1 specific binding sites was significantly increased (34%) in the frontal cortex of the treated group. Also D-2 specific binding sites in the frontal cortex was significantly decreased (56%) in AF64A-treated group. The specific binding sites of neither D-1 nor D-2 were changed in the hippocampus of treated group.

DISCUSSION

The present results demonstrate that the activity of the central dopaminergic neuronal activities are changed after the icv infusion of AF64A. Although the levels of DA and metabolites in each brain regions are differently changed, the ratios of DOPAC/DA are decreased in the striatum and hippocampus. The activity of TH in frontal cortex is decreased, while that of MAO-B in striatum is increased. The specific binding sites of D-1 and D-2 dopamine receptors are increased in the frontal cortex.

It has been reported that AF64A, irreversible neurotoxin, impaired the choline uptake in the cholinergic nervous terminals (Chrobak *et al.*, 1988; Nakahara *et al.*, 1988). However the changes in dopaminergic neuronal activities were somewhat controversially reported by AF64A treatment; either no changes in the parameters of dopaminergic neuronal activities (Smith, 1988) or decreases in DA levels and metabolites (Hortnagl *et al.*, 1987; Meana *et al.*, 1992). The present results indicate that dopaminergic neuronal activities are decreased by the icv infusion of AF64A. Thus, the present results suggest that the administration of AF64A affect the central dopaminergic- as well as cholinergic neuronal activities. Furthermore the decreased dopaminergic activities are differently affected at each regions by the icv infusion. The DOPAC/DA ratios in striatum and hippocampus are decreased, which indicate the decreased turnover of DA, while the levels of DA and DOPAC in frontal cortex are decreased without the ratios unchanged. The decrease of DA levels in frontal cortex could be partly due to a decreased synthesis of DA. This might be partly supported by the decreased activities of TH in frontal cortex as the present results. However, Dawson *et al.* (1990) reported that AF64A lesions resulted in no alteration in striatal TH activity. It has been reported that TH activities might be affected by the various factors, such as the release of DA (Galloway *et al.*, 1986), the DA nerve activities (Quik and Sourkes, 1977) and the released DA (Wolf *et al.*, 1986). Since TH activities are not affected either with the increase of DA levels in hippocampus or without the changes in DA levels in striatum, the changes in the turnover of DA in these regions might be due to the changes in the utilization process of DA, such as the release and the uptake of DA in dopaminergic nervous terminal.

Monoamine oxidase (MAO) is a well known enzyme associated with central monoamine transmitter systems. Also it has been reported that MAO was two activities, called MAO-A and MAO-B (Strolin-Benedetti and Dostert, 1985). It has been reported that MAO-B activities were age-dependently increased in various areas of brain regions, such as the nigrastriatal systems (Strolin-Benedetti and Keane, 1980), frontal cortex and hippocampus (Amenta *et al.*, 1994). The increases in MAO-B activities after the AF64A treatment as the present results is similar to those after the aged. It has been reported that L-deprenyl, a potent MAO-B inhibitor, enhances cognitive function in aged-rats (Brandeis *et al.*, 1991). Although the exact cause and consequences in the increased MAO-B activities are not known, the present results suggest that AF64A might be useful for the cognitive animal model. However it has been reported that the localized MAO acitivites within and outside dopaminergic synaptosomes are different (Stenstrom *et al.*, 1987). Therefore the changes in intra- and extrasynaptosomal MAO-B activities are needed to further studied.

The various changes in dopaminergic receptors have been reported in Alzheimer disease; either unaltered (Cross *et al.*, 1984) or reduced (Rinne *et al.*, 1986). Also striata lesioned with AF64A induced a reduction in D2 dopamine receptors (Dawson *et al.*, 1990). Although the present results reveals the reduction in striatal D1 dopamine receptors, the specific D1 and D2 dopamine receptors in frontal cortex are increased. Although the exact reason for the different alterations of dopamine receptors in each brain regions is unknown, it is possible to be either the different neuronal connections between areas or the different characteristic of dopamine receptors. It has been reported that a close interaction between cholinergic and dopaminergic neuron exists in the brain (DeBellerche and Bradford, 1980; Westerink and Korf, 1976). It is well known that the chronic administration of either agoinsts or antagonists induce the down- and up-regulations of its receptors. Therefore the increased D1 and D2 dopamine receptors in frontal cortex might be due to the reduced DA levels in frontal cortex as the present results. Although the various parameters of central dopaminergic neuronal systems are changed by the AF64A treatment, the behavioral

interactions between dopamine agents and the AF64A-induced animals are remained to be further elucidated.

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