Effects of Bifemelane on Muscarinic Receptors and Choline Acetyltransferase in the Brains of Aged Rats Following Chronic Cerebral Hypoperfusion Induced by Permanent Occlusion of Bilateral Carotid Arteries

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Received December 22, 1995 Accepted June 28, 1996

ABSTRACT—Cerebral hypoperfusion was chronically induced in aged rats via permanent bilateral occlusion of common carotid arteries (2VO). Marked reduction of the B_max value of the muscarinic receptors (mAChR) in both the cortex and striatum and the V_max value of choline acetyltransferase (ChAT) activity in the cortex, hippocampus and striatum were observed as compared with those of control aged rats. No significant changes in mAChR and ChAT activity were observed between young control rats and young 2VO rats. One month post-surgery in aged rats, daily doses of bifemelane (10 mg/kg) or aniracetam (50 mg/kg) were administered orally over a 4-week period. Administration of bifemelane significantly increased B_max values and decreased apparent K_d values for 3H-quinuclidinyl benzilate (QNB) in mAChR in the striatum. Chronic administration of bifemelane or aniracetam also enhanced ChAT activity in the cortex, hippocampus and striatum. In particular, administration of bifemelane resulted in a significant increase in V_max values of ChAT in all three brain regions, while no significant change in K_m values for ChAT was observed. These results suggest that bifemelane is responsible for this activity, thereby enhancing the functioning system of CNS cholinergic neurons of cerebral hypoperfused aged rats.

Keywords: Aged rat, Permanent bilateral occlusion of common carotid arteries, Muscarinic receptor, Choline acetyltransferase, Bifemelane, Aniracetam

A number of drugs and substances have been developed as means to decrease the symptoms of memory impairment observed in patients with senile dementia, and their effects have been studied in animal models (1–5). The results of these experiments, however, have not yet yielded sufficient data to be useful in the clinical setting. Various kinds of animal models have been developed to study human dementia; these include drug- (2), brain lesion- (6–8), or transient ischemia- (3) induced amnesia in rodents.

We previously reported that the investigation of brain cholinergic systems in aged rats could provide information concerning age-related disorders in humans and that these data should aid in establishing a model of senile dementia that could be used to investigate potentially therapeutic drugs (9, 10). This model, however, employed rats that were simply aged, not true models of senile dementia.

It has been reported that cerebral blood flow is decreased in patients with senile dementia, such as in the case of Alzheimer's disease (11, 12). Senile dementia is characterized by marked cognitive impairments that parallel the progression of neuronal damage (13). Permanent bilateral occlusion of common carotid arteries (2VO) in rats is used as a model for brain ischemia (14). In addition, it is known that permanent 2VO induces chronic cerebral hypoperfusion (15).

In the present study, we examined whether chronic cerebral hypoperfusion induced by permanent 2VO in aged rats would result in neuronal damage and observed the subsequent muscarinic receptor (mAChR) concentration and choline acetyltransferase (ChAT) activity. We also investigated the effect of long-term administration of bifemelane on cholinergic markers in aged 2VO rats; bifemelane is known to be an effective treatment for cerebrovascular disease in clinical trials as well as in animal models. The results were then compared to those obtained with aniracetam (16), which is also considered
a candidate for treating the dementia associated with Alzheimer's disease.

MATERIALS AND METHODS

Animals

We used male, Sprague-Dawley rats (Seiwa Laboratory Animals, Inc., Fukuoka) aged 6 weeks (young) and 24 months (aged). The rats were housed in a controlled environment (25±2°C, 50±5% humidity), with light between 7:00 and 19:00 hours, and food and water available ad libitum. Chronic cerebral hypoperfusion was produced in both young and aged rats by tying off the common carotid arteries (2VO) under ether anesthesia. Sham-operated rats, in which the cervical region had been removed, but the common carotid arteries remained intact, were used as controls. After 4 weeks of permanent 2VO, the rats were divided into four groups: young control rats, young 2VO rats, aged control rats and aged 2VO rats. To determine the in vivo effects of bifemelane or aniracetam on muscarinic receptors and ChAT activity, daily doses of bifemelane hydrochloride (10 mg/kg) or aniracetam (50 mg/kg) were administered orally to aged 2VO rats over a period of 4 weeks. Aged rats receiving aniracetam (50 mg/kg) were administered orally to aged 2VO rats over a period of 4 weeks. Aged rats receiving physiological saline orally for 4 weeks served as controls.

Under ether anesthesia, all rats were sacrificed by decapitation 24 hr after the final dose. The brain of each rat was quickly removed from the skull and dissected into three parts: the cortex, which includes the forebrain and temporal, parietal, and occipital lobes (combined for all assays); the hippocampus; and the striatum. In order to minimize variations among individual subjects, tissues from at least five rats were pooled for use in each experiment and stored at -80°C.

Muscarinic receptor binding assay

Sample brain tissue from each subject was homogenized in 5 vol. (wt./vol.) of 50 mM Tris-HCl buffer (pH 7.5). The homogenates were centrifuged at 900 x g for 10 min. The supernatants were again centrifuged at 12,000 x g for 20 min. Pellets were resuspended in 10 mM Tris-HCl buffer containing 5 mM MgSO4 (pH 7.5) and adjusted to a protein concentration of 1.0 mg/ml, yielding a crude synaptosomal fraction. The apparent dissociation constant (Kd) and the density (Bmax) of mAChR in the crude synaptosomal fraction were assayed according to the method of Yamamura and Snyder (17) using [3H]-quinuclidinyl benzilate ([3H]-QNB) as the specific ligand. Aliquots of the crude synaptosomal fraction (100 µl, 1.0 mg protein/ml) were incubated in a total volume of 1.0 ml at 37°C for 30 min with various concentrations of [3H]-QNB (0.04–0.6 nM) in 10 mM Tris-HCl buffer containing 5 mM MgSO4. After incubation, 4 ml of ice-cold 10 mM Tris-HCl buffer containing 145 mM NaCl (pH 7.5) was added to each of the aliquots, and the bound ligand was separated from the free ligand by rapid filtration through GF/B glass filters (Whatman). The filter papers were washed four times with the same ice-cold buffer. The filters were allowed to dry overnight, and then 10 ml of Triton X-100-toluene scintillation fluid was added to each. Samples were measured by a liquid scintillation spectrometer with an efficiency of 47%. Specific binding was defined as the difference between measurements taken in the presence and absence of atropine (1 µM). Binding data derived from the saturation experiments were analyzed by computerized linear regression analysis to estimate the apparent Kd and Bmax. We applied [3H]-QNB at a concentration of 0.04 nM for experiments investigating the effects of bifemelane or aniracetam on muscarinic receptors in vitro.

Choline acetyltransferase activity assay

Choline acetyltransferase (ChAT) activity was determined by means of a minor modification of the radio metric method of Fonnum (18) using [3H]-acetyl coenzyme A (acetyl CoA) as a substrate. Each brain tissue sample was homogenized in 5 vol. of ice-cold 50 mM phosphate buffer (pH 7.4) containing 10 mM EDTA and 2.5% Triton X-100. These preparations were allowed to stand for 15 min at 4°C and were then centrifuged at 20,000 x g for 10 min. The protein content in the resulting supernatant was adjusted to 1 mg/ml with 50 mM phosphate buffer (pH 7.4), and this sample was used for the ChAT analysis. The incubation solutions contained 0.2 mM [3H]-acetyl CoA, various concentrations of choline bromide (1–10 mM), 300 mM NaCl, 0.1 mM physostigmine and 20 mM EDTA in 50 mM phosphate buffer (pH 7.4). Eight microliters of the enzyme preparation and 20-µl aliquots of the incubation solution were combined and incubated at 37°C for 15 min. The reaction was terminated by adding 1 ml of cold 10 mM phosphate buffer (pH 7.4) and 300 µl of 0.5% Kalibor in acetonitrile. The reaction products were extracted with 2 ml of toluene. We mixed samples of the extract with Triton X-100-toluene scintillation fluid and measured their radioactivities by liquid scintillation spectrometry. Values of enzymatic activities were calculated from the dpm values and expressed as nanomoles of acetylcholine synthesized/min/mg protein. Values of the apparent Km and Vmax were obtained from Lineweaver-Burk plots of specific activity as a function of choline bromide concentration.

Protein determination

Protein concentrations were determined according to the method of Lowry et al. (19) with bovine serum albu-
min used as the standard.

**Chemicals**

[^H]-QNB (2.22–3.22 TBq/mmol) was purchased from New England Nuclear (Boston, MA, USA) and[^H]-acetyl CoA (111–122 GBq/mmol) was purchased from Amersham (Buckinghamshire, England). Atropine sulfate, acetyl CoA, choline bromide, p-chloro-mercuribenzoic acid (PCMB) and physostigmine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Kalibor was obtained from Dojindo Laboratories (Kumamoto). All other chemicals were obtained from Wako Pure Chemical Industries Ltd. (Osaka). Bifemelane hydrochloride and aniracetam were donated by the Eisai Co., Ltd. (Tokyo).

**Statistics**

The results are expressed as the mean or mean±S.E. for five rats. The statistical significance of the differences between young and aged rats, control and bifemelane-treated rats, control and aniracetam-treated aged rats was determined by Student’s t-test.

**RESULTS**

**Muscarinic receptors in young and aged 2VO rat brains**

To determine whether mAChR in the central nervous system are affected by permanent 2VO, we performed kinetic analysis of[^H]-QNB binding in three parts (cortex, hippocampus, striatum) of young and aged rat brains. As shown in Table 1, there is a significant age-related reduction in the apparent $K_d$ in the cortex, striatum and hip-

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<th>Table 1. Comparison of kinetic parameters of muscarinic receptors in young and aged control and young and aged 2VO rat brains</th>
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After 4 weeks of permanent 2VO, the rats were administered orally doses of bifemelane (10 mg/kg) or aniracetam (50 mg/kg) over a period of 4 weeks. All rats were sacrificed by decapitation 24 hr after the final dose. Binding data were derived from saturation analysis. Binding for each concentration of radioligand was measured in triplicate, and non-specific binding was defined as the binding in the presence of 1 pM atropine, also performed in triplicate. The apparent $B<sub>max</sub>$ and $K_d$ for[^H]-QNB binding were determined graphically by Scatchard analysis and are expressed as the mean±S.E. of the values obtained for five rats. control: no treatment, 2VO: permanent bilateral common carotid occlusion. $B<sub>max</sub>$: pmol/mg protein, $K_d$: pM. *P<0.05, compared with young (control), †P<0.05, compared with young (2VO), ′P<0.05, compared with aged (control).

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<th>Table 2. Comparison of kinetic parameters of ChAT activity in young and aged control and young and aged 2VO rat brains</th>
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After 4 weeks of permanent 2VO, the rats were administered orally doses of bifemelane (10 mg/kg) or aniracetam (50 mg/kg) over a period of 4 weeks. All rats were sacrificed by decapitation 24 hr after the final dose. Each value is expressed as the mean±S.E. for five rats. $V<sub>max</sub>$ and $K_m$ values were determined from Lineweaver-Burk double reciprocal plots of values obtained from graphic representations of the kinetic data. control: no treatment, 2VO: permanent bilateral common carotid occlusion. $K_m$: mM, $V<sub>max</sub>$: nmol/min/mg protein. *P<0.05, compared with young (control), †P<0.05, compared with young (control), ′P<0.05, compared with young (2VO), †P<0.05, compared with aged (control).
pocampus. It also shows an age-related decrease in the apparent B\text{max} for \(^3\)H-QNB in the cortex and striatum between control young and aged rats.

After treating young and aged rats with permanent 2VO, we discovered a significant age-related decrease in B\text{max} in the cortex and striatum between young and aged 2VO rats. We found no difference, however, in the kinetic parameters in all three regions between control young rats and young 2VO rats. We found a significant increase in the K\text{d} in all three regions as well as a slight reduction in the B\text{max} between control aged rats and aged 2VO rats.

ChAT in young and aged 2VO rats

Table 2 shows that with advancing age, the V\text{max} value of ChAT declined significantly in the cortex, hippocampus and striatum. We saw no significant age-related differences, however, in the K\text{m} values. After treating young and aged rats with permanent 2VO, we observed a significant decrease in V\text{max} values in all three regions of the brain. However, we saw no difference in the kinetic parameters of ChAT between control young rats and young 2VO rats; Only the K\text{m} values of the hippocampus and striatum varied. We found a slight decrease in the V\text{max} in each of the three regions of aged 2VO rats and a

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Fig. 1. Effect of long-term administration of bifemelane or aniracetam on B\text{max} and K\text{d} values for \(^3\)H-QNB binding in the three regions of aged 2VO rat brains. Binding data were derived from saturation analysis. The binding for each concentration of radioligand was measured in triplicate, and non-specific binding was defined as the binding in the presence of 1 \(\mu\)M atropine, also determined in triplicate. B\text{max} values were determined graphically by Scatchard analysis and are expressed as the mean ± S.E. of the values obtained for five rats. ■: saline-treated aged 2VO rats, ■: bifemelane-treated aged 2VO rats, ■: aniracetam-treated aged 2VO rats. *P < 0.05, compared with saline-treated aged 2VO rats.
significant decrease in the $K_m$ in the hippocampus and striatum of aged 2VO rats as compared with that of control aged rats.

Effects of long-term administration of bifemelane and aniracetam on mAChR

Figure 1 compares resulting $B_{max}$ values representing $^3$H-QNB binding in three different brain regions of control aged 2VO rats (administered with saline), aged 2VO rats after oral administration of bifemelane (10 mg/kg/day) and aged 2VO rats after administration of aniracetam (50 mg/kg/day) for 4 weeks. The only significant increase in $B_{max}$ value that we observed was in the striatum of bifemelane-treated, aged 2VO rats as compared with control aged 2VO rats. There was a significant decrease in $K_d$ in the striatum of bifemelane-treated, aged 2VO rats (Fig. 1, lower).

Effects of long-term administration of bifemelane and aniracetam on ChAT

$V_{max}$ values for ChAT were higher in the striatum than in either the cortex or hippocampus. Administration of bifemelane for 4 weeks resulted in a significant increase in the $V_{max}$ values of ChAT in the three regions of bifemelane-treated, aged 2VO rats as compared with those of control aged 2VO rats. Treatment with aniracetam
resulted in an increased $V_{\text{max}}$ value of ChAT only in the cortex of aged 2VO rats (Fig. 2, upper). In contrast, the $K_m$ value of ChAT was lower in the hippocampus and striatum than in the cortex. No change in $K_m$ values was noted in either bifemelane- or aniracetam-treated aged 2VO rats following long-term administration of bifemelane or aniracetam (Fig. 2, lower).

**Effects of bifemelane and aniracetam on in vitro $^3$H-QNB binding in young and aged 2VO rat brain**

The effects of bifemelane and aniracetam on $^3$H-QNB binding sites in the three regions of young and aged 2VO rat brains were compared in vitro. As shown in Fig. 3, $^3$H-QNB binding sites were moderately displaced with increasing concentrations of bifemelane. The displacement curve paralleled the typical curve of pirenzepine, a known antagonist of muscarinic receptors. Treatment with aniracetam, however, did not alter $^3$H-QNB binding. These displacement curves in aged 2VO rats were similar to those in young 2VO rats. Moreover, there was no difference among displacement curves representing the three regions of young and aged rat brains.

**In vitro effects of bifemelane and aniracetam on ChAT activity in young and aged 2VO rat brain**

We investigated the effects of both bifemelane and aniracetam on ChAT activity in the three regions of young and aged 2VO rats. It is known that PCMB inhibits ChAT activity, and Figure 4 shows a marked inhibition of ChAT activity. Neither bifemelane nor aniracetam, however, inhibited ChAT activity in any of the three regions of young and aged 2VO rat brains.

**DISCUSSION**

It has been reported that cerebral blood flow (CBF) is decreased in patients with senile dementia associated with Alzheimer's disease and that this is accompanied by
marked cognitive impairments. The reports suggest that chronic hypoperfusion may have an important role in the progression of dementia (11, 12). It has also been reported that chronic mild hypoperfusion induced by permanent 2VO caused learning and memory deficits in rats and that this progressive cognitive deficit paralleled the progression of neuronal damage (13, 15). Because it is well-known that the functioning cholinergic system can be damaged by cerebral ischemia (14), we compared various cholinergic biochemical markers (mAChR and ChAT activity) in the cortex, hippocampus and striatum of the brains of aged (24 month) and young (6 weeks) rats treated with permanent 2VO. We previously reported that the investigation of brain cholinergic systems in aged control rats could provide useful information pertaining to age and age-related disorders in humans (9, 10). However, in this study, ChAT activity and the number of mAChR in aged 2VO rats decreased nearly 10–20% as compared with those in aged control rats. These observations are more important since 2VO-induced cerebral hypoperfusion in aged rats provides an important animal model of cholinergic dysfunction in patients with senile dementia associated with cerebrovascular dementia. Although in the present study, no significant change in these cholinergic markers was observed between young permanent 2VO rats and young control rats, it has been reported that permanent 2VO can cause learning deficits within 1 month after the 2VO operation and the progressive cognitive deficits that follow permanent 2VO parallel the progression of neuronal damage (15). The reason for the differences in these data is not clear. In general, it is known that the bodies of young rats have high resiliency, enabling them to quickly recover from invasive surgery. So, the reductions of cholinergic markers in young permanent 2VO rats might not be observed since the cholinergic functions were compensated by the other healthy parts of the brain.

This animal model is advantageous for examining whether drugs can prevent the neuronal damage induced by chronic cerebral hypoperfusion. As such, we investigated whether long-term treatment with bifemelane could halt or slow the decrease of cholinergic function caused

Fig. 4. Effect of bifemelane, aniracetam or PCMB on ChAT activity in young and aged 2VO rats in vitro. An enzyme solution of each young and aged brain part was incubated with the substrate mixture for 15 min at 37°C with various concentrations of bifemelane, aniracetam or PCMB. Each point represents the mean value of the ChAT activity determined in three separate experiments. Top: young 2VO rat, bottom: aged 2VO rat. ○: bifemelane, ▲: aniracetam, ●: PCMB.
by 2VO in aged rats. In general, in vivo manipulations that enhance receptor activity, such as the administration of cholinomimetics or inhibitors of acetylcholinesterase, decrease receptor concentration. Conversely, chronic pharmacological blockade of receptors increases receptor levels. In this study, the long-term treatment of aged 2VO rats with bifemelane caused a significant increase in the density of mAChR in the striatum, as determined by measuring $^3$H-QNB binding. It has been reported that bifemelane improves the impaired working memory caused by both scopolamine and cerebral ischemia (20). Bifemelane also augments the effect of physostigmine, and the effects of bifemelane are antagonized by atropine (21). Thus, bifemelane appears to be a partial agonist for mAChR.

Since $^3$H-QNB binding was displaced with increasing concentrations of bifemelane (Fig. 3) and the apparent $K_a$ values for $^3$H-QNB decreased (Fig. 1, lower), we suggest that bifemelane may possess some antagonistic action or direct action on mAChR. It is interesting that long-term administration of bifemelane to aged 2VO rats reversed the 2VO-induced deficit in the number of mAChR. The mechanism underlying the bifemelane-induced increase in mAChR remains unknown and requires further study. While aniracetam in this study did not affect mAChR in aged 2VO rats, both in vivo and in vitro, it has been reported that the long-term administration of aniracetam (16) or a related compound, nebracetam (22), caused a decrease in the number of M$_1$-mAChR in the rat hippocampus.

The cholinergic system is important in learning and memory, and its function declines with normal aging. ChAT activity is generally accepted as a good indicator of cholinergic function, as well as the degree of severity of senile dementia, including Alzheimer's disease and cerebrovascular dementia (23-25). A number of drugs and substances have been tested for possible improvement of the memory impairment symptoms seen in patients with senile dementia. Pantoyl-GABA (26) is reported to increase both the release of acetylcholine and the quantity of ChAT. Long-term administration of bifemelane, idebenone or propentofylline is also reported to enhance ChAT activity in the aged rat brain (27). In this study, long-term administration of bifemelane or aniracetam to aged 2VO rats also significantly increased $V_{\text{max}}$ values of the permanent 2VO-induced deficit of ChAT activity, but not $K_m$ values of ChAT for its substrates. In addition, neither drug altered ChAT activity in vitro. In general, ChAT activity depends on the concentration of its substrates, choline and acetyl CoA (18). Moreover, the concentration of both precursors of acetylcholine, choline and acetyl CoA, are known to be affected by the level of CBF (28).

Cerebral ischemia causes a marked reduction of brain ATP and phosphocreatine levels in rats (29). Both bifemelane and aniracetam have previously been reported to be effective in promoting the uptake of glucose into the brain and subsequently increasing the levels of choline and acetyl CoA (30, 31). These drugs also have been shown to prevent decreases in acetylcholine levels that ordinarily result from ischemia (20, 32). From these findings, it appears likely that the increase of ChAT activity in aged 2VO rat brains is due to increases in the levels of its substrates, choline and acetyl CoA, by the long-term administration of bifemelane or aniracetam, although the mechanism leading to the increased $V_{\text{max}}$ values is unclear.

Since bifemelane appears to slow the 2VO-induced decrease in cholinergic markers in the brain of aged rats, this agent may be considered a candidate for the clinical examination of the cholinergic hypothesis of senile dementia, including Alzheimer's disease and cerebrovascular dementia.

REFERENCES