EFFECTS OF CARNOSINE ON AMYGDALOID-KINDLED SEIZURES IN SPRAGUE–DAWLEY RATS

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Abstract—The effects of carnosine (β-alanyl-L-histidine) on amygdaloid-kindled seizures were investigated in rats. I.p. injection of carnosine (500, 1000, 1500 mg/kg, i.p.) significantly decreased seizure stage, afterdischarge duration and generalized seizure duration, and significantly prolonged generalized seizure latency of amygdaloid-kindled seizures, in a dose-dependent, and time-related manner. The protecive effect of carnosine (1500 mg/kg) was completely antagonized by histamine H1-antagonists pyrilamine (2, 5 mg/kg, i.p.) and diphenhydramine (5, 10 mg/kg, i.p.), but not by histamine H2-antagonist zolantidine even at a high dose of 10 mg/kg. Carnosine (1500 mg/kg, i.p.) caused a significant increase of carnosine and histidine levels in the hypothalamus, thalamus, hippocampus, amygdala and cortex, as well as histamine levels in the hippocampus and amygdala. I.c.v. injection of α-fluoromethylhistidine (50 μg, i.c.v.), a selective and irreversible histidine decarboxylase inhibitor, only partially reversed the inhibition of amygdaloid-kindled seizures induced by carnosine. In addition, carnosine significantly decreased glutamate contents in the amygdala and hippocampus. These results indicate that carnosine could protect against amygdaloid-kindled seizures in rats, and its action may be due to the activation of histamine postsynaptic H1-receptors via two different mechanisms, one being carnosine’s direct action, and the other being indirectly mediated by histaminergic pathway. The study suggests that carnosine may be an endogenous anticonvulsant factor in the brain and could be used as a new antiepileptic drug in the future. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: β-alanyl-L-histidine, epilepsy, glutamate, histamine, histamine H1-receptors.

Epilepsy is one of the most common neurological disorders and characterized by recurrent seizures, afflicting about 1% of the population (Berg et al., 1996). Clinical data demonstrated that long-term epilepsy, especially among children, or ingesting conventional antiepileptic drugs over time is likely to result in cognitive deficits (e.g. memory or attention problems) as well as other CNS side effects such as psychomotor speed abnormalities, somnolence, asthenia, and dizziness (Kwan and Brodie, 2001). New drug therapy has been expected.

A number of studies suggest that the histaminergic neuron system plays an important role in the pathogenesis of seizure disorders. The brain histamine seems to be involved in mechanisms regulating seizure susceptibility, and a possible anticonvulsant action of histamine has been well documented (Scherkl et al., 1991; Kamei et al., 1998; Zhang et al., 2003b). An i.c.v. injection of histamine or i.p. injection of histidine increases threshold for amygdaloide kindling and pentylene tetrazol-induced seizures (Kamei et al., 1998; Chen et al., 2002; Okuma et al., 2001). We also reported that the seizure development induced by pentylenetetrazol was facilitated in histidine decarboxylase (HDC) deficient mice compared with that in wild-type mice (Chen et al., 2003). Therefore, certain histaminergic substances have been expected as clinical anticonvulsants (Vohora et al., 2001; Zhang et al., 2003a). However, histamine cannot penetrate across the blood–brain barrier (BBB) and it has been reported to be involved in brain inflammation (Silverman et al., 2000). The histamine released from mast cells, platelets and basophils potentiates the neurogenic inflammation, and induces increased vascular permeability and BBB rupture (Abbott, 2000). Recently Vizuete et al. (2000) reported that the infusion of histamine into rat substantia nigra results in an acute inflammatory response manifested by a loss of glial fibrillary acidic protein-immunolabeled astrocytes.

Carnosine (β-alanyl-L-histidine), a natural occurring dipeptide, was first discovered by Gulewitsch and Amiradzibi (1900) in meat extract in 1900. Carnosine presents in muscle and brain of mammals in high concentrations (O’Dowd et al., 1988; Biffo et al., 1990; Bonfanti et al., 1999), and it has been characterized as a putative neurotransmitter in the olfactory receptor neurons (Bonfanti et al., 1999). However, so far, the understanding about the roles of carnosine in the brain is very limited. On the other hand, carnosine serves as a reservoir for histidine, which is a precursor of histamine (Kasziba et al., 1988; Flancbaum et al., 1990). There seem to be certain relations between carnosine and histamine, and it is proposed that carnosine might be a new histaminergic drug and could be used in clinical therapeutics instead of histamine. For example, like histamine, carnosine could inhibit the hyperglycemia induced by the injection of 2-deoxy-D-glucose into the lateral cerebral ventricle through regulating autonomic nerves via histamine H3 receptor (Yamano et al., 2001). Yet, few reports have demonstrated the significant relations be-
between brain carnosine and histamine. And there is only limited information about the effects of carnosine on amygdaloid-kindled seizures, which is an adequate animal model of human complex partial epilepsy with secondary generalization (Albright and Burnham, 1980).

Therefore, the objectives of our investigations are designed to elucidate the pharmacological mechanisms of the effects of carnosine on amygdaloid-kindled seizures in rats.

**EXPERIMENTAL PROCEDURES**

**Animals**

All experiments were carried out in accordance with the ethical guidelines of the Zhejiang University Animal Experimentation Committee and was in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Furthermore, attempts were made to minimize the number of animals used in the study and their suffering. The animals used in this study were male Sprague–Dawley rats (♂, 220–300 g, Gradell, Certificate No. 22–9601018, Experimental Animal Center, Zhejiang University, China), maintained in individual cages with a 12-h light/dark cycle (lights on from 08:00–20:00 h). Water and food were given ad libitum. Experiments were carried out each day between 10:00–17:00 h.

**Surgery**

Under sodium pentobarbital anesthesia (35 mg/kg, i.p., Abbott, North Chicago, IL, USA), the rats were fixed on a stereotaxic apparatus (Narishige, SR-5, Tokyo, Japan), and electrodes (0.2 mm in diameter) were implanted into the right basolateral amygdala according to the following coordinates derived from the atlas of Paxinos and Watson (1998): AP: −2.4 mm, L: −4.8 mm, V: −8.8 mm. The electrodes were bipolar twisted stainless steel Teflon-coated wires (tip distance of 0.5–1.0 mm, A.M. Systems. Inc., WA, USA) and insulated except for 0.5 mm at the tip. Electrodes were connected to a miniature receptacle, which was embedded in the skull with dental cement. A guide cannula made of stainless steel tubing 700 μm in outer diameter, was implanted into the right lateral ventricle according to the following coordinates measured from bregma: AP: −1.0 mm, L: −1.5 mm, V: −3.8 mm from the skull. At least 10 days were allowed for recovery from surgery.

**Experimental procedures in kindled seizures**

Stimulation of the amygdala was applied with a constant electric stimulator (SEN-7203, SS-202J; Nihon Kohden, Tokyo, Japan), and electroencephalograms (EEGs) at the amygdala were recorded with the PowerLab system (AD Instruments, NSW, Australia). The tissue samples were analyzed by HPLC-combined with an electrochemical detector, using the technique developed in our laboratory for the simultaneous and sensitive analysis of carnosine, histidine, histamine, glutamate and GABA. The system consists of model 582 pump, model 540 autosampler and four-channel CoulArray electrochemical detector. The HPLC was controlled and the data acquired and analyzed using CoulArray® software. All of the above equipments were from ESA (Chelmsford, MA, USA). After reacting with the derivate o-phthalaldehyde, analytes were separated on a 3 μm, 3×50 mm Capcell Pak MG C18 column from Shiseido (Tokyo, Japan). A two-component gradient elution system was used, with component A of the mobile phase being 100 mM Na2HPO4, 13% acetonitrile, and 22% methanol, pH 6.8, and component B being similar to A except with 5.6% acetonitrile and 9.4% methanol. A gradient elution profile was used as follows: 0–3.5 min, isocratic 100% B; 3.5–20 min, linear ramp to 0% B; 20–30 min, isocratic 0% B; 22–23 min, linear ramp to 100% B; 23–30 min, isocratic 100% B. The flow rate was set to 0.75 mL/min. The temperature of the column was maintained at 38 °C. The first cell was set at ±250 mV, whereas the second cell was set at ±350 mV. All standards were obtained from Sigma (St. Louis, MO, USA). Under these conditions, the retention time of glutamate, histidine, carnosine, GABA and histamine is 5.58, 8.70, 10.84, 15.16 and 18.36 min, respectively. The detection limits (signal/noise=3) were 0.1 μg for glutamate, 5 ng for histidine, carnosine and GABA, and 1 ng for histamine. Reproducibility of the present method was assessed from a series of standard mixture six times a day for four consecutive days. The average coefficients of variation of within-day and between-day assays were 0.5% to 2.3% and 0.7% to 3.6%, respectively, for all analyzed substances.
Drugs

The drugs used were carnosine (Sigma), pyrilamine dihydrochloride (Sigma), diphenhydramine hydrochloride (Sigma), zolantidine dimaleate (SmithKline Beecham, London, UK), and α-fluoromethylhistidine (α-FMH, Merck Sharp & Dohme Research Laboratory, Rahway, NJ, USA). α-FMH was dissolved in sterilized saline and injected i.c.v. in a fixed volume of 5 μL over a period of 60 s at a constant speed with a continuous infusion pump (KN-201, Natsume, Tokyo, Japan). The other drugs were dissolved in saline, and were injected i.p. Drugs were administered once a week. The same animals were repeatedly used, and they experienced all doses of each drug administered in an ascending order.

Histology

At the end of experiment, the animals were killed, and the localizations of guide cannula and electrodes were verified histologically. Only animals with guide cannula located in the lateral ventricle and electrodes lying within the basolateral amygdala were included in the following analysis.

Statistical analysis

All data are expressed as the mean±S.E.M. Statistical analysis was carried out using SPSS 11.5 for Windows. One-way analysis of variance (ANOVA) with Dunnett’s test was used for calculating the statistical significance. In the case of brain glutamate and GABA contents, Mann-Whitney’s U test was used. Statistical significance was set at \( P<0.05 \).

RESULTS

Effect of carnosine on amygdaloid-kindled seizures in rats

I.p. injection of carnosine decreased seizure stage, shortened both AD duration and generalized seizure duration, and prolonged generalized seizure latency of amygdaloid-kindled seizures in a dose-related and time-related manner (Fig. 1) (Fig. 2). Carnosine at a dose of 200 mg/kg produced no significant inhibition of amygdaloid-kindled seizures. At a dose of 500 mg/kg it significantly decreased...
seizure stage, and shortened AD duration and generalized seizure duration 1 h after injection. Carnosine at a dose of 1000 mg/kg significantly decreased seizure stage and AD duration, prolonged generalized seizure latency 0.5, 1 and 2 h after injection, and significantly shortened generalized seizure duration 1 and 2 h after injection. And at a dose of 1500 mg/kg, a significant inhibition was observed from 0.5–2 h after injection in all parameters of amygdaloid-kindled seizure. Additionally, carnosine at doses used did not produce any significant effect on animal behaviors such as locomotor activity (data not shown).

Effects of histamine H1- and H2-antagonists on the protective effect induced by carnosine on amygdaloid-kindled seizures in rats

The effects of histamine H1- and H2-antagonists on the protective effect induced by carnosine (1500 mg/kg, i.p.) on amygdaloid-kindled seizures were shown in Fig. 3. I.p. treatment with pyrilamine, a selective central histamine H1-antagonist, antagonized the protective effect of carnosine dose-dependently. Pyrilamine at doses of 2 and 5 mg/kg significantly reversed the carnosine-induced inhibition of amygdaloid-kindled seizures in rats (P<0.01). Similarly, diphenhydramine, another histamine H1-antagonist, at doses of 5 and 10 mg/kg also significantly reversed the effects of carnosine in a dose-dependent manner (P<0.05). On the other hand, pretreatment with zolantidine, a selective central histamine H2-antagonist did not antagonize the protective action of carnosine even at a high dose of 10 mg/kg. Additionally, administration of pyrilamine, diphenhydramine, or zolantidine at the above doses alone produced no appreciable effect on all parameters of amygdaloid-kindled seizure in rats (data not shown).

Effect of α-FMH on the protective action induced by carnosine on amygdaloid-kindled seizures in rats

I.c.v. treatment with α-FMH, a selective and irreversible HDC inhibitor, slightly reversed carnosine-induced anti-convulsive effect (Fig. 4). α-FMH at a dose of 50 μg significantly antagonized the descent of seizure stage induced by carnosine (P<0.05), and also showed a tendency to reverse the inhibition of AD duration induced by carnosine (1500 mg/kg, i.p.).

Fig. 3. Effects of histamine H1- and H2-antagonists on the inhibition of amygdaloid kindled seizures induced by carnosine. Histamine H1- and H2-antagonists were injected i.p. 30 min before electrical stimulation. Stimulation was performed 1 h after injection of carnosine (1500 mg/kg, i.p.). Each value represents the mean±S.E.M. of eight to 10 rats. ** P<0.01 represent statistically significant difference as compared with saline group. * P<0.05 and ## P<0.01 represent statistically significant difference as compared with the carnosine + saline group.
carnosine. However, no marked effect of α-FMH was observed on the protective action of carnosine on generalized seizure latency and duration at all doses used in the study.

Effect of i.p. injection of carnosine on brain carnosine, histidine, and histamine levels

The time-course changes of brain carnosine, histidine, and histamine levels after i.p. injection of carnosine (1500 mg/kg) are shown in Fig. 5. I.p. injection of saline did not alter brain levels of carnosine, histidine and histamine at any time during the experiment. Therefore, their levels were pooled to calculate a single baseline concentration for each analyte. Compared with these controls, i.p. injection of carnosine produced a significant increase of carnosine levels in the hypothalamus, thalamus, hippocampus, amygdala and cortex from 0.5–2 h after drug injection. The greatest increase of brain carnosine levels occurred after 1 h. Four hours after injection, increased brain carnosine levels were almost restored to the control levels (Fig. 5A). Similarly, a remarkable increase of histidine levels was observed in the hypothalamus and amygdala from 0.5–2 h, as well as in the thalamus, hippocampus and cortex from 1 to 2 h after carnosine injection (P<0.05). However, there was no significant change of histamine levels in the hypothalamus, thalamus and cortex (Fig. 5C).

Effect of i.p. injection of carnosine on brain glutamate and GABA levels

I.p. injection of carnosine (1500 mg/kg) caused a significant decrease of glutamate levels in the hippocampus (25.1%) and amygdala (38.1%). On the other hand, carnosine treatment did not significantly influence GABA levels in brain (Table 1).

DISCUSSION

In the present study, we firstly found that carnosine had a significant protection against amygdaloid-kindled seizures in rats. Carnosine markedly decreased seizure stage, shortened AD duration and generalized seizure duration, and prolonged generalized seizure latency, which suggests that carnosine has an inhibitory effect on the secondary generalization of kindled seizures. In addition, it has been demonstrated that carnosine could easily penetrate across BBB and has few side effects (Matsukura and Tanaka, 2000). Therefore, it is likely that carnosine might be a new potential anticonvulsant drug for clinical therapy of human complex partial epilepsy in future.
Fig. 5. Levels of carnosine, histidine, and histamine in the different regions of the rat brain at 0.5, 1, 2 and 4 h after i.p. injection of carnosine (1500 mg/kg). Each value represents the mean±S.E.M. of six to eight rats. Levels of controls, depicted at 0 h, are the mean of analyte levels from all rats (n=2 at each time) given saline (see Results). * P<0.05 and ** P<0.01 represent statistically significant difference as compared with control group.
The protection of carnosine was significantly antagonized by histamine H1-agonists pyrilamine and diphenhydramine, but not by histamine H2-agonist zolantidine. Carnosine is a precursor of histidine (Kasziba et al., 1988; Flancbaum et al., 1990). In the present study, we found that carnosine produced a significant increase in histamine levels in the hippocampus and amygdala. These findings suggest that the protective effect of carnosine may be mediated by activating histaminergic system and histamine post synaptic H1-receptors. Brain histamine acts as an endogenous anticonvulsant substance, and histamine H1-receptor plays an important role in regulating seizure susceptibility (Okuma et al., 2001; Zhang et al., 2003a,b). A marked increase in the hippocampal H1-receptor binding has been observed around the epileptic foci of complex partial seizure in order to prevent the spread of seizure activity (Inuma et al., 1993; Toyota et al., 1999). Several histamine H1-agonists including diphenhydramine and chlorpheniramine increase focal and general epileptic discharges among both epileptic patients and rodents (Yokoyama and Inuma, 1996; Kamei et al., 2000). Moreover, we also found that α-FMH, a selective and irreversible HDC inhibitor also significantly antagonized carnosine-induced inhibition of amygdaloid-kindled seizure stage. Therefore, these results provided more evidence that the protection of carnosine against amygdaloid-kindled seizure was due to an increase in histamine synthesis, and histamine H1-receptor might play a critical role in the protective mechanism of carnosine.

On the other hand, we are surprised to find that α-FMH could only partially reversed the anticonvulsant effect of carnosine, though Kamei et al. (1993) have reported that α-FMH (50 μg, i.c.v) could significantly decrease brain histamine contents to about 50% as compared with control. Our previous study also proved α-FMH could effectively decrease histamine contents in rat brain (Chen et al., 1999). In addition, at 0.5 h after carnosine injection when histamine levels in the hippocampus and amygdala was not significantly elevated, carnosine also produced a significant anticonvulsant effect. These data suggest that the elevated brain histamine levels in the amygdala and hippocampus induced by carnosine might not be the only mechanism contributing to the protection of carnosine against amygdaloid-kindled seizures, and there might exist other potential stronger mechanisms. Recently, O’Dowd and Miller (1998) reported that like histamine, carnosine activates the smooth muscle histamine H1-receptors directly to provoke vasoconstriction with greater efficacy than noradrenaline. The agent-receptor binding test also found that carnosine could directly bind to histamine H1-receptors in CNS (O’Dowd and Miller. 1998). Therefore, it is likely that through directly acting at histamine H1-receptors and provoking a histamine-like response, carnosine could also significantly inhibit amygdaloid-kindled seizures in rats. Furthermore, compared with the mechanism of elevating brain histamine levels, the mechanism of directly acting at histamine H1-receptors might be predominant for the protection of carnosine against amygdaloid-kindled seizures in rats.

We also interestingly found that α-FMH had not any inhibitory effect on the protective action of carnosine on generalized seizure latency and duration. The amygdaloid-kindled seizure stages can be divided into two forms: focal (limbic) seizure (stages 1–3) and generalized (motor) seizure (stages 4–5) (Loscher and Schmidt, 1988). The generalized seizure latency and duration are two indexes of generalized seizure characterized by BFC, which rises in the case of propagation of focal seizure activity to cortical areas, basal ganglia and brainstem (Kelly et al., 1999). In contrast to carnosine which significantly increased in the whole brain, histamine levels were significantly elevated only in the hippocampus and amygdala where epileptic activity originates and focal seizure depends (Loscher and Ebert, 1996). It suggests that the increased histamine levels may mainly participate in the inhibition of focal seizure, whereas the increased carnosine levels may have a more extensive and stronger protection for both focal and generalized seizure. Therefore, α-FMH could slightly antagonize the decrease of seizure stage and AD duration induced by carnosine due to its inhibition of histamine synthesis, while the protection of generalized seizure induced by carnosine could not be reversed by α-FMH because it was unable to reverse the protection induced by carnosine via its directly activating histamine H1-receptor.

In the present study, we found that the contents of glutamate in the hippocampus and amygdala were significantly increased following the increase of histamine levels after carnosine injection. As a principal excitatory neurotransmitter, glutamate plays important roles in the initiation, spread and maintenance of epileptic activity of both epileptic patients and kindled rodents (Meldrum, 1992; Bradford, 1995). Compounds directly or indirectly modifying the normal or pathologic release of glutamate can also

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**Table 1. Effect of carnosine on brain GABA and glutamate contents in rats**

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>GABA contents (μg/g tissue)</th>
<th>Glutamate contents (μg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Carnosine</td>
</tr>
<tr>
<td>Cortex</td>
<td>32.1±1.8</td>
<td>37.0±2.8</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>565.5±26.8</td>
<td>529.4±39.7</td>
</tr>
<tr>
<td>Thalamus</td>
<td>192.2±15.1</td>
<td>179.6±8.5</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>160.1±16.4</td>
<td>128.0±10.4</td>
</tr>
<tr>
<td>Amygdala</td>
<td>262.8±31.3</td>
<td>204.4±40.0</td>
</tr>
</tbody>
</table>

Carnosine was i.p. injected (1500 mg/kg) 1 h before rats were killed for analysis. Values are means±S.E.M. (n=6).

* P<0.05 represents statistically significant difference as compared to control group.
alter seizure express of animal model and man (Meldrum, 1994; Parsons et al., 1998). It is interesting that the most obvious changes of contents of glutamate occurred in these same brain regions as histamine, suggesting that the increased histamine contents might suppress the activity of glutaminergic system. However, the contents of GABA, the principal inhibitory neurotransmitter in the brain, did not significantly changed after injection of carnosine. Therefore, it would be proposed that the anticonvulsant effect of carnosine may partially be mediated by the decline of glutamate contents in the hippocampus and amygdala.

Previous studies have demonstrated that carnosine (via histidine) could be metabolically transformed into histamine in muscle and kidney (Greene et al., 1984; Flancbaum et al., 1990; Fitzpatrick et al., 1991). However, whether the metabolic carnosine–histidine–histamine pathway also exists in the CNS has not yet been reported. In the present study, carnosine caused a significant increase of carnosine and histidine levels in the hypothalamus, thalamus, hippocampus, amygdala and cortex, while histamine levels were significantly elevated only in the amygdala and hippocampus. These results provide more evidences to support that carnosine could also be metabolically transformed into histamine in the CNS. On the other hand, we interestingly found that the increase in histidine levels in the hypothalamus, thalamus and cortex did not further increase histamine levels simultaneously in the corresponding brain regions. We have no more data to explain why carnosine produced the surprising discrepancy among different brain region. The most likely reason would be that carnosine produces an inhibition on the activity of HDC (Sakamoto et al., 1985), and this action appears brain region-dependent. In addition, it would be also plausible to argue that there is still another metabolic pathway of carnosine rather than the carnosine–histidine–histamine pathway in the brain. Further biochemical studies are needed to elucidate the precise metabolic pathway of carnosine in the brain.

CONCLUSION

In summary, our experiment preliminarily provides the evidence that carnosine has a significant anticonvulsant effect on amygdaloid-kindled seizures in rats. The mechanism of the protective effect of carnosine may involve at least two different (direct and indirect) pathways: pathway I, directly acting at histamine H1-receptors and provoking a histamine-like response; pathway II, indirectly activating histamine H1-receptors after metabolic transformation into histamine. The present study suggests that carnosine may be an endogenous anticonvulsant factor in the brain and necessitates its further study as a new efficient antiepileptic drug in the future.

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