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European Journal of Pharmacology 524 (2005) 84–88
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Nitric oxide involves in carnosine-induced hyperactivity in chicks

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Received 6 September 2005; accepted 8 September 2005
Available online 19 October 2005

Abstract

Carnosine has been characterized as a putative neurotransmitter and implicated as having a possible role in neuron–glia cell interactions. We previously confirmed that central administration of carnosine induced hyperactivity in chicks. In the present study, we investigated the effects of nitric oxide (NO) synthase (NOS) inhibitors on carnosine-induced hyperactivity in chicks. Carnosine-induced (3.2 μmol) hyperactivity was attenuated by intracerebroventricular (i.c.v.) co-administration with a non-selective NOS inhibitor N^G-nitro-L-arginine methyl ester HCl (200 and 400 nmol) in a dose-dependent manner, while the hyperactivity was not attenuated by the inactive isomer of the NOS inhibitor N^G-nitro-D-arginine methyl ester HCl (400 nmol). The i.c.v. injection of a selective inhibitor of inducible NOS (iNOS) L-N^6-(1-iminoethyl) lysine HCl (400 nmol) did not affect carnosine-induced hyperactivity. These results suggest that carnosine-induced hyperactivity may be linked to the constitutive NOS (cNOS), rather than iNOS, in the brain. Central carnosine may regulate brain function and/or behaviors by NO generation via cNOS in chicks.

Keywords: Carnosine; Hyperactivity; Nitric oxide; Nitric oxide synthase; Chick

1. Introduction

Carnosine (β-alanyl-L-histidine) has been characterized as a putative neurotransmitter in olfactory receptor neurons (Bonfanti et al., 1999), and was implicated as having a possible role in neuron–glia cell interactions (Marchis et al., 2000). In the brain, studies on localization of carnosine and its derivative anserine (β-alanyl-1-methyl-L-histidine) show a complex pattern of expression that involves both neuronal and glial cell types. The glial localization, widely distributed throughout the whole brain and spinal cord, includes a subset of mature astrocytes and oligodendrocytes, whereas neuronal localization is restricted to a particular type of neurons (the olfactory receptor neurons) and to a restricted population of putative migrating neurons and neuroblasts (Marchis et al., 2000).

Nitric oxide (NO) is a gaseous mediator that transmits signals between cells or from one part of a cell to another. NO synthase (NOS) catalyses the conversion of L-arginine to NO. The generated NO is soluble in both water and lipid, and diffuses freely within and between cells. It has a half-life of only a few seconds and is rapidly inactivated upon contact with haemoglobin (Vallance, 2003). NO has diverse physiological functions in various tissues such as neurotransmission, vasodilation, reduction of oxidative stress, induction of apoptosis, antiapoptotic action, pro-inflammatory effects, and anti-inflammatory properties (Abramson et al., 2001; Thomas, 2000; Vallance, 2003). NOS can be found ubiquitously in the brain including neuron and glia. The enzymatic activity seems to vary depending on the brain region (Forstermann et al., 1990; Wiesinger, 2001).

We previously demonstrated that intracerebroventricular (i.c.v.) injection of carnosine induced hyperactivity in chicks and hypothesized that the hyperactivity might represent one of the roles of carnosine in neuron–glia interactions (Tomonaga et al., 2004). On the other hand, Alaghband-Zadeh et al. (2001) suggested that carnosine has NO generative effect via NOS in the liver in vitro. Thus, carnosine-induced hyperactivity may be linked to NO generation via NOS in the brain. To clarify the function of central carnosine, therefore, we investigated the

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doi:10.1016/j.ejphar.2005.09.008

effects of NOS inhibitors on carnosine-induced hyperactivity in chicks.

2. Materials and methods

2.1. Animals

Day-old male chicks (Julia strain) were purchased from a local hatchery (Murata Hatchery, Fukuoka, Japan). The chicks were reared as a group maintained in a room with continuous lighting, and temperature at 30±1 °C. Food (Toyohashi Feed and Mills, Aichi, Japan) and water were freely accessible. The chicks were placed individually in cages and allowed to habituate for 1 day before beginning the experiments. All birds used were 5 or 6 days old. The birds were distributed into experimental groups based on their body weight so that the average body weight of each group was as uniform as possible. Experimental procedures followed the guidance for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government.

2.2. Drugs and i.c.v. injection

Carnosine, N^G-nitro-L-arginine methyl ester HCl (L-NAME), N^G-nitro-D-arginine methyl ester HCl (D-NAME) and L-N^6-(1-iminoethyl) lysine HCl (L-NIL) were purchased from Sigma (St. Louis, MO). Drugs were dissolved in 0.85% saline containing a 0.1% Evans Blue solution and administered i.c.v. in a volume of 10 μl using a microsyringe according to the method of Davis et al. (1979). We did not use anesthetization for injection, since the stress suffered by this method is minimal (Koutoku et al., 2005). The volume applied was determined by the previous study (Tomonaga et al., 2004). Control groups were given the saline solution. We used an acrylic device to hold the head of chicks. The head holder with a hole in the head plate of the device was accommodated for the 26-gauge needle of a Hamilton microsyringe into the lateral ventricle, and a drug was intracerebroventricularly injected by the syringe. The injection depth was approximately 0.6 cm from the bottom of the head plate. At the end of the experiment, the birds were sacrificed with an overdose of sodium pentobarbital and the brains removed to verify injection location. Data from individuals not having Evans Blue dye present in the lateral ventricle were deleted.

2.3. Behavioral tests

In Experiment 1, the effect of a non-selective NOS inhibitor L-NAME on hyperactivity induced by carnosine was investigated in chicks. Chicks were injected i.c.v. with saline (control), L-NAME (400 nmol), carnosine (3.2 μmol) or carnosine (3.2 μmol) plus L-NAME (400 nmol). After injection, the birds were placed in a monitoring cage (40×30×20 cm) and video recorded for 15 min. The chicks were not habituated to the activity cages before the experiment. Spontaneous activity was measured by infrared beam sensor (NS-AS01; Neuroscience, Inc. Japan) placed about 20 cm above the monitoring cage. The sensor detected the movement of the chicks based on the released infrared ray associated with the temperature of the birds. The data were analyzed by the software of the digital data recording system (DAS-008; Neuroscience, Inc. Japan) and the counts outputted from the software were defined as spontaneous activity.

Experiment 2 was done to investigate the dose-dependent effect of L-NAME on carnosine-induced hyperactivity in chicks. Birds were injected i.c.v. with saline (control), carnosine (3.2 μmol), carnosine (3.2 μmol) plus L-NAME (200 nmol) or carnosine (3.2 μmol) plus L-NAME (400 nmol). Spontaneous activity was analyzed as described in Experiment 1.

In Experiment 3, we investigated the effect of the inactive isomer of the NOS inhibitor, D-NAME, on carnosine-induced hyperactivity in chicks. Chicks were injected i.c.v. with saline (control), D-NAME (400 nmol), carnosine (3.2 μmol) or carnosine (3.2 μmol) plus D-NAME (400 nmol). Spontaneous activity was analyzed as described in Experiment 1.

Finally, Experiment 4 was done to investigate the effect of a selective inducible NOS (iNOS) inhibitor L-NIL on carnosine-induced hyperactivity in chicks. Chicks were injected i.c.v. with saline (control), L-NIL (400 nmol), carnosine (3.2 μmol) or carnosine (3.2 μmol) plus L-NIL (400 nmol). Spontaneous activity was analyzed as described in Experiment 1.

2.4. Statistical analysis

Data were statistically analyzed by three-way repeated analysis of variance (ANOVA) with respect to treatment and time in Experiments 1, 3 and 4. In Experiment 2, data were analyzed by two-way repeated ANOVA, and when significant interaction was detected, comparisons between means were made using the Tukey–Kramer's test. Statistical analysis was conducted using a commercially available package StatView (version 5, SAS Institute, Cary, U.S.A. 1998). The results are presented as means±S.E.M.

3. Results

3.1. Effect of L-NAME on carnosine-induced behaviors in chicks

Fig. 1 shows the effect of L-NAME on carnosine-induced spontaneous activity in chicks. The effect of carnosine on cumulative spontaneous activity was significant ($F(1,25)=17.93$, $P<0.001$). An interaction between time and carnosine was also significant ($F(2,50)=8.854$, $P<0.0005$), indicating that cumulative spontaneous activity in the birds treated with carnosine increased with time compared with saline and L-NAME treatments. An interaction between carnosine and L-NAME was not significant ($F(1,25)=2.68$, $P=0.1142$). There was not a significant interaction among time, carnosine and L-NAME ($F(2,50)=2.01$, $P=0.1447$). These two interactions imply that carnosine-induced hyperactivity tended to be attenuated by L-NAME but the effect was not significant.

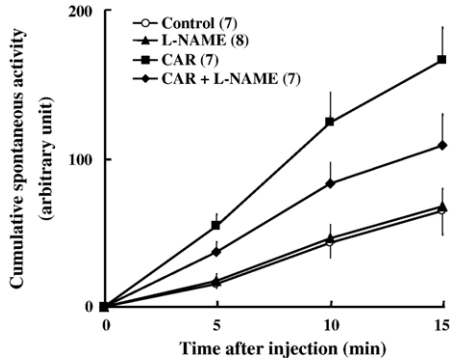

Fig. 1. Effect of N^G-nitro-L-arginine methyl ester HCl on carnosine-induced spontaneous activity for 15 min in chicks. Figures in parentheses indicate the number of chicks in each group. CAR and L-NAME indicate carnosine and N^G-nitro-L-arginine methyl ester HCl, respectively. Values are cumulative number of spontaneous activity per 5 min. Values are presented as means±S.E.M.

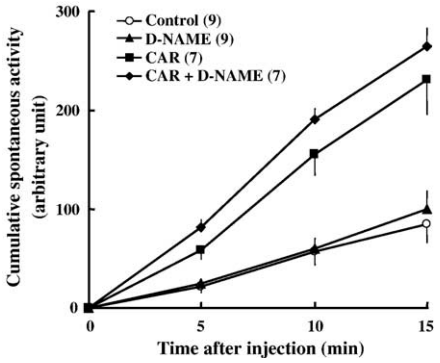

Fig. 3. Effect of N^G-nitro-D-arginine methyl ester HCl on carnosine-induced spontaneous activity for 15 min in chicks. Figures in parentheses indicate the number of chicks in each group. CAR and D-NAME indicate carnosine and N^G-nitro-D-arginine methyl ester HCl, respectively. Values are cumulative number of spontaneous activity per 5 min. Values are presented as means±S.E.M.

3.2. Dose dependent effects of L-NAME on carnosine-induced behaviors in chicks

Fig. 2 demonstrates dose-dependent effects of L-NAME on carnosine-induced spontaneous activity in chicks. The effect of drugs on cumulative spontaneous activity was significant ($F(3,27)=7.964$, $P<0.001$). An interaction between time and drugs was also significant ($F(6,54)=7.511$, $P<0.0001$). These results imply that the difference in activity between carnosine or carnosine plus L-NAME treatment and the control diverged with time. L-NAME clearly inhibited carnosine-induced hyperactivity in a dose-dependent manner.

3.3. Effect of D-NAME on carnosine-induced behaviors in chicks

Fig. 3 shows the effect of D-NAME on carnosine-induced spontaneous activity in chicks. The effect of carnosine on cumulative spontaneous activity was significant ($F(1,28)=56.027$, $P<0.0001$). Interaction between time and carnosine was also significant ($F(2,56)=34.579$, $P<0.0001$). These results indicate that cumulative spontaneous activity in the birds treated with carnosine greatly increased with time compared with saline and D-NAME treatments. On the other hand, an interaction between carnosine and D-NAME ($F(1,28)=0.674$, $P=0.4185$) was not significant. This suggests that carnosine-induced hyperactivity was not attenuated by D-NAME.

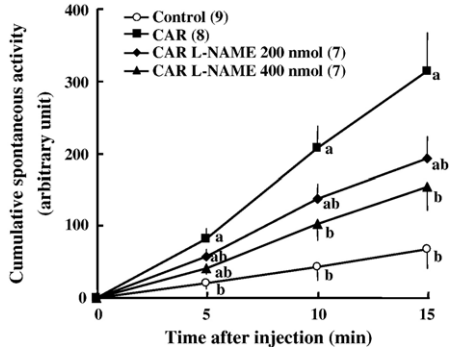

Fig. 2. Dose-dependent effects of N^G-nitro-L-arginine methyl ester HCl on carnosine-induced spontaneous activity for 15 min in chicks. Figures in parentheses indicate the number of chicks in each group. CAR and L-NAME indicate carnosine and N^G-nitro-L-arginine methyl ester HCl, respectively. Values are cumulative number of spontaneous activity per 5 min. Values with a different letter at each time are significantly different at $P<0.05$. Values are presented as means±S.E.M.

3.4. Effect of L-NIL on carnosine-induced behaviors in chicks

Fig. 4 represents the effect of L-NIL on carnosine-induced spontaneous activity in chicks. The effect of carnosine on cumulative spontaneous activity was significant ($F(1,24)= 76.411$, $P<0.0001$). An interaction between time and carnosine was also significant ($F(2,48)=23.168$, $P<0.0001$). These results indicate that cumulative spontaneous activity in birds treated with carnosine greatly increased with time compared with saline and L-NIL treatments. On the other hand, an interaction between carnosine and L-NIL ($F(1,24)=2.402$, $P=0.1343$) was not significant. These results suggest that carnosine-induced hyperactivity was not influenced by L-NIL.

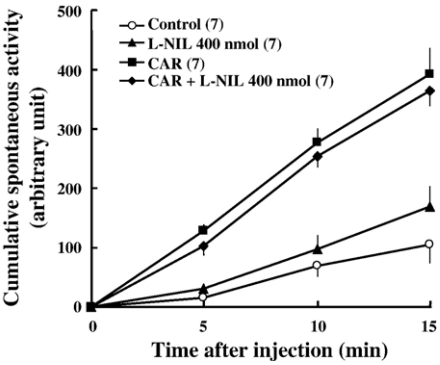

Fig. 4. Effect of L-N^6-(1-iminoethyl) lysine HCl on carnosine-induced spontaneous activity for 15 min in chicks. Figures in parentheses indicate the number of chicks in each group. CAR and L-NIL indicate carnosine and L-N^6-(1-iminoethyl) lysine HCl, respectively. Values are cumulative number of spontaneous activity per 5 min. Values are presented as the means±S.E.M.

4. Discussion

In the present study, there is so much variability in the data across the experiment. For an interpretation of the phenomenon, we hypothesized that because chicks in one experiment was a little different from chicks in another experiment in terms of the season when they were born and/or on the environment the experiment was conducted, the sensitivity of birds to carnosine differs across the experiment. However, the effect of carnosine is reproducible. Thus, we think that our experimental condition is reliable for the pharmacological study of central carnosine.

We demonstrated that a non-selective NOS inhibitor L-NAME could attenuate carnosine-induced hyperactivity (Experiment 1) and the effect was dose-dependent (Experiment 2). Because the inactive isomer of the NOS inhibitor, D-NAME, did not attenuate carnosine-induced hyperactivity in Experiment 3, the attenuation of carnosine-induced hyperactivity by L-NAME might be due to the specific inhibition of NOS activity in the brain. Therefore, the results of the present study suggested that carnosine-induced hyperactivity may be linked to NO generation via NOS in the brain.

NOS isoforms are either constitutively expressed NOS (e.g. neuronal ncNOS, endothelium ecNOS) or iNOS. Constitutively expressed NO synthases (cNOSs) produce picomolar–nanomolar amounts of NO for short periods in response to receptor stimulation or shear stress. In contrast to the ecNOS and ncNOS isoforms, iNOS is expressed following exposure to diverse stimuli such as inflammatory cytokines and lipopolysaccharide (LPS), and it generates significantly greater and more sustained amounts of NO when compared to the cNOS (Abramson et al., 2001). For example, iNOS protein in the lung was not detected 3 h after the intraperitoneal injection of the iNOS inducer LPS in mice, but the iNOS protein did appear 6 h after the injection (Lim et al., 2004). From these results, in contrast to cNOS, NO derived from iNOS did not appear to be generated immediately after the stimulation. On the other hand, carnosine-induced hyperactivity was an acute effect because the effect could be observed within 5 min and the acute effects were suppressed by L-NAME in Experiments 1 and 2. Therefore, carnosine-induced hyperactivity may be linked to cNOS rather than iNOS. To clarify this speculation, we investigated the effect of a selective iNOS inhibitor L-NIL on carnosine-induced hyperactivity in Experiment 4. The dose of L-NIL in the experiment was the same amount for L-NAME to inhibit carnosine-induced hyperactivity in Experiments 1 and 4. We found that L-NIL did not affect carnosine-induced hyperactivity. Even though L-NAME was widely used as a non-selective NOS inhibitor, it had a tendency to inhibit cNOS selectively rather than iNOS (Bryk and Wolff, 1999). Furthermore, L-NIL inhibited iNOS stronger than L-NAME (Chlopicki et al., 1999). Therefore, carnosine-induced hyperactivity may be linked to the stimulation of cNOS rather than iNOS.

Thomas (2000) suggested that drugs that enhance production of NO via cNOS would be beneficial in the treatment of neurodegenerative diseases. Beneficial effect of carnosine in the brain could be partly explained by its effect on NO generation via cNOS. Hoffmann et al. (1996) suggested that carnosine uptake in cultures of rat glial cells was restricted to astrocytes. Centrally administered carnosine in the present study might be taken up by astrocytes. Since cNOS exists in astrocytes (Ma et al., 1994), it can be hypothesized that carnosine in the present study might have stimulated NO production via cNOS in astrocytes. On the other hand, it can also be hypothesized that centrally administered carnosine might directly and/or indirectly affect neurons, and as a result, activate cNOS in neurons. We cannot specify the precise site of action of injected carnosine from the present results alone. Further study has to be done to clarify whether carnosine directly and/or physiologically affect NOS in the brain. For example, because the relationship between the localization of carnosine and NOS in CNS pathways has not been well studied, it has to be investigated in the future. On the other hand, widely used to inhibit NOS activity, L-NAME acted as not only a NOS inhibitor but also as a muscarinic receptor antagonist (Buxton et al., 1993). However, the muscarinic receptor antagonist scopolamine attenuated the decrease of spontaneous activity in chicks (Koutoku et al., 2005) and i.c.v. injection of scopolamine induced hyperactivity in rats (Katner et al., 1996). From these previous reports, we hypothesized that muscarinic receptor antagonism itself would not attenuate hyperactivity in chicks. Therefore, attenuation of carnosine-induced hyperactivity by L-NAME might be mainly caused by the inhibition of cNOS. In any case, we suggested that central carnosine might regulate brain function and/or behaviors by NO generation via cNOS in chicks. Further study is needed to clarify physiological relationships between carnosine and NO in the brain.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science and Uehara Memorial Foundation.

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