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Carnosine has antinociceptive properties in the inflammation-induced nociceptive response in mice

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ABSTRACT

Carnosine is a biologically active dipeptide that is found in fish and chicken muscle. Recent studies have revealed that carnosine has neuroprotective activity in zinc-induced neural cell apoptosis and ischemic stroke. In the present study, we examined the expression of carnosine in the spinal cord, and the antinociceptive potency of carnosine in a mouse model of inflammation-induced nociceptive pain. Immunohistochemical studies with antiserum against carnosine showed an abundance of carnosine-immunoreactivity in the dorsal horn of the mouse spinal cord. Double-immunostaining techniques revealed that carnosine was expressed in the neurons and astrocytes in the spinal cord. Oral administration of carnosine attenuated the number of writhing behaviors induced by the intraperitoneal administration of 0.6% acetic acid. Treatment with carnosine also attenuated the second phase, but not the first phase, of the nociceptive response to formalin. Moreover, intrathecal, but not intraplanter, administration of carnosine attenuated the second phase of the nociceptive response to formalin. Our immunohistochemical and behavioral data suggest that carnosine has antinociceptive effects toward inflammatory pain, which may be mediated by the attenuation of nociceptive sensitization in the spinal cord.

1. Introduction

Carnosine (β-alanyl-L-histidine) is an endogenous dipeptide that is expressed in many tissues, including the central nervous system (Bonfanti et al., 1999; De Marchis et al., 2000). Carnosine has been shown to have both antioxidative and anti-inflammatory effects, and to act as a free-radical scavenger and an organic pH buffer (Boldyrev et al., 1999, 2004; Bonfanti et al., 1999; Trombley et al., 2000). It also protects against acute renal failure caused by ischemia/reperfusion in rats (Kurata et al., 2006). Moreover, treatment with carnosine attenuates NMDA-induced excitotoxic injury in differentiated PC12 cells through its conversion to histidine and histamine (Shen et al., 2007).

While carnosine was shown to be widely expressed in glial cells throughout the whole brain and spinal cord, limited expression was observed in a particular type of neurons (olfactory receptor neurons) and in particular migrating neurons and neuroblasts (Biffo et al., 1990). Pathophysiolgical conditions could affect the expression pattern of carnosine in the central nervous system. Indeed, while carnosine was not found in the cerebral cortex under normal conditions, the expression of carnosine in astrocytes was enhanced after permanent middle cerebral artery occlusion (pMCAO; Rajanikant et al., 2007). Moreover, exogenous carnosine attenuated the effects of pMCAO through an increase in glial glutamate uptake (Shen et al., 2010).

Carnosine is safe, well-tolerated and commonly used as a dietary supplement. It is a natural water-soluble antioxidant that suppresses the generation of reactive oxygen species and scavenges lipid peroxidation products during free radical reactions both in vitro and in vivo (Holliday and McFarland, 2000; Prokopieva et al., 2000). Carnosine also protects neural cells against glutamate-induced toxicity (Shen et al., 2010). A chronic pain model showed increased superoxide production and glutamate release in the spinal cord. Moreover, it has also been reported that the spinal expression of glial glutamate transporter (GLT-1) was down-regulated after sciatic nerve-ligation and inflammation (Maeda et al., 2008). These findings led us to speculate that treatment with carnosine may ameliorate the nociceptive response. The present study was undertaken to examine if carnosine produces antinociception in a model of inflammatory pain.

2. Materials and methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Kyushu University of Health and Welfare, as adopted by the Committee on Animal Research of Kyushu University of Health and Welfare, which is

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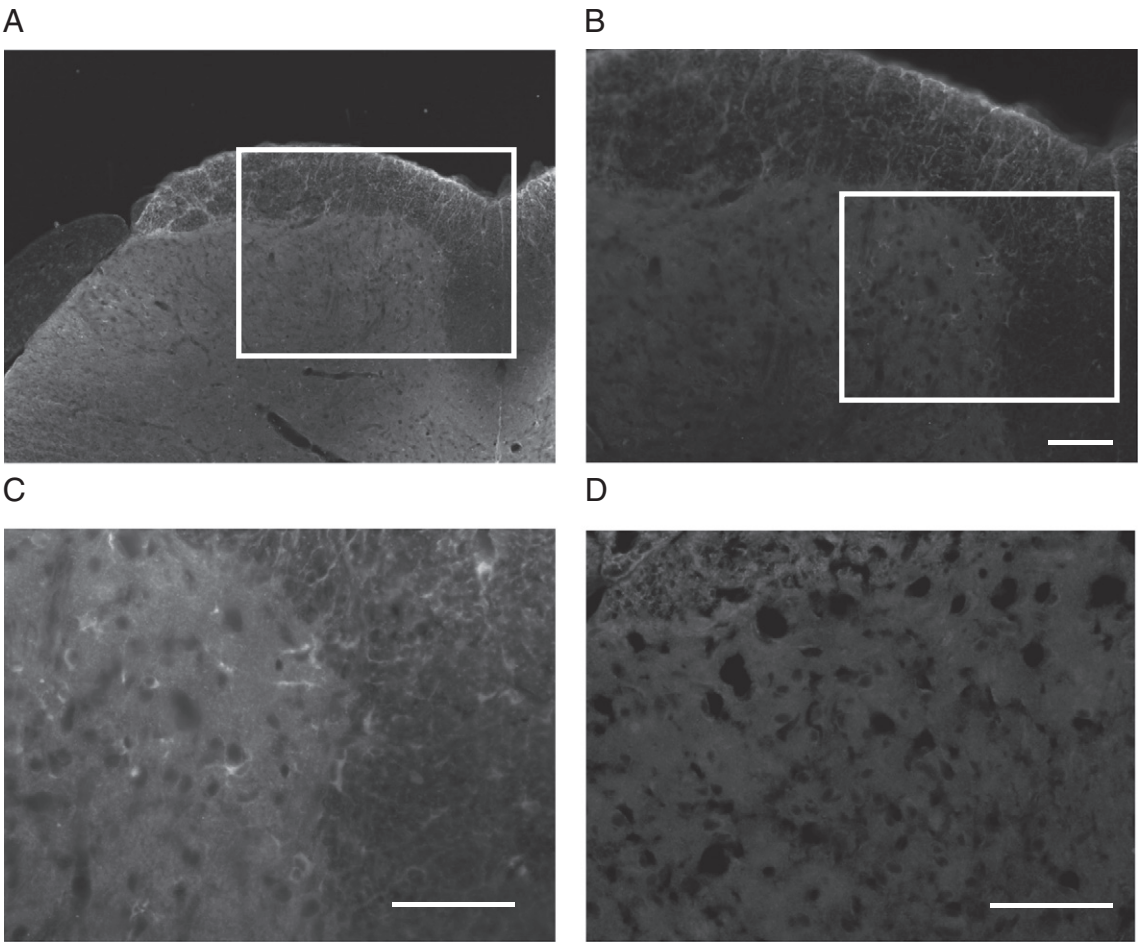

Fig. 1. Photomicrographs of mouse lumbar spinal sections labeled with carnosine-antisera or carnosine-antisera preabsorbed with the peptide overnight. A. Low-magnification image showing carnosine-immunoreactive cells are expressed in laminae I and II of the dorsal horn; deeper laminae express much fewer carnosine-immunoreactive cells. B. Higher magnification of the area outlined in A where carnosine-immunoreactive cells are concentrated. C. Higher magnification of the area outlined in B. D. A section of the lumbar spinal cord labeled with carnosine-antisera pre-absorbed with the peptide at 10 μg/ml overnight; immunoreactivity is not seen in this section. Scale bar: 100 μm for A. and B.; 50 μm for C and D.

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2.1. Animals

Male ICR mice (Kyudo Laboratory Animals, Inc., Saga, Japan) weighing 20 to 30 g were used in this study. Animals were housed five per cage in a room maintained at 23 ± 0.5 °C with an alternating 12-h light–dark cycle. Food and water were available ad libitum. Animals were used only once in all of the experiments.

2.2. Immunohistochemistry

Mice were anesthetized with pentobarbital (60 mg/kg, i.p) and intracardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The spinal cords were removed and thick coronal sections containing L4, L5 and L6 were initially dissected. These sections were postfixed in the same fixative for 2 h, immersed in 30% sucrose/PBS solution overnight, and then frozen in an embedding compound (Sakura Finetechnical, Tokyo Japan). Frozen 8 μm-thick coronal sections were cut with a freezing microtome (REM-700; Yamato Kohki Industrial Co., Saitama, Japan) and thaw-mounted on poly-l-lysine-coated glass slides.

The lumbar spinal cord sections were blocked in 10% normal goat serum in 0.1 M PBS for 2 h at room temperature. Each primary antibody was diluted in 0.1 M PBS containing 0.5% bovine serum albumin and 0.4% Triton-X 100 (1:1000 rabbit polyclonal antibodies against carnosine) and was incubated for 2 days at 4 °C. The sections were then rinsed and incubated with the secondary antibody conjugated with Alexa 488 for 2 h at room temperature. The slides were then coverslipped with fluorescence mounting medium (Dako, Carpinteria, CA). Fluorescence of immunolabeling was detected using a fluorescence microscope (AxioImager A1; Carl Zeiss, Germany).

In control experiments, spinal sections were processed with carnosine-antisera that had been pre-absorbed with the peptide at 10 μg/ml overnight.

For double-labeling, sequential labeling with primary antibodies from different hosts was used. Tissues were first blocked with 10% normal goat serum and then incubated in carnosine antiserum (1:1000 dilution with 0.4% Triton X-100 and 1% bovine serum albumin in PBS) for 48 h in a cold room with gentle agitation. After several washes with PBS, the sections were rinsed and incubated with the secondary antibody conjugated with Alexa 488 for 2 h at room temperature. After being thoroughly rinsed with PBS for 2 h, tissues were blocked with normal horse serum and incubated in NeuN (1:1000 dilution; Chemicon International, Inc., Temecula, CA) or GFAP (Chemicon International Inc., Temecula, CA) for 48 h in a cold room with gentle agitation. After washing with PBS for 30 min, tissues were incubated with secondary antibody conjugated with Alexa 592 for 2 h at room temperature. Finally, tissues were washed for 30 min with PBS. The slides were

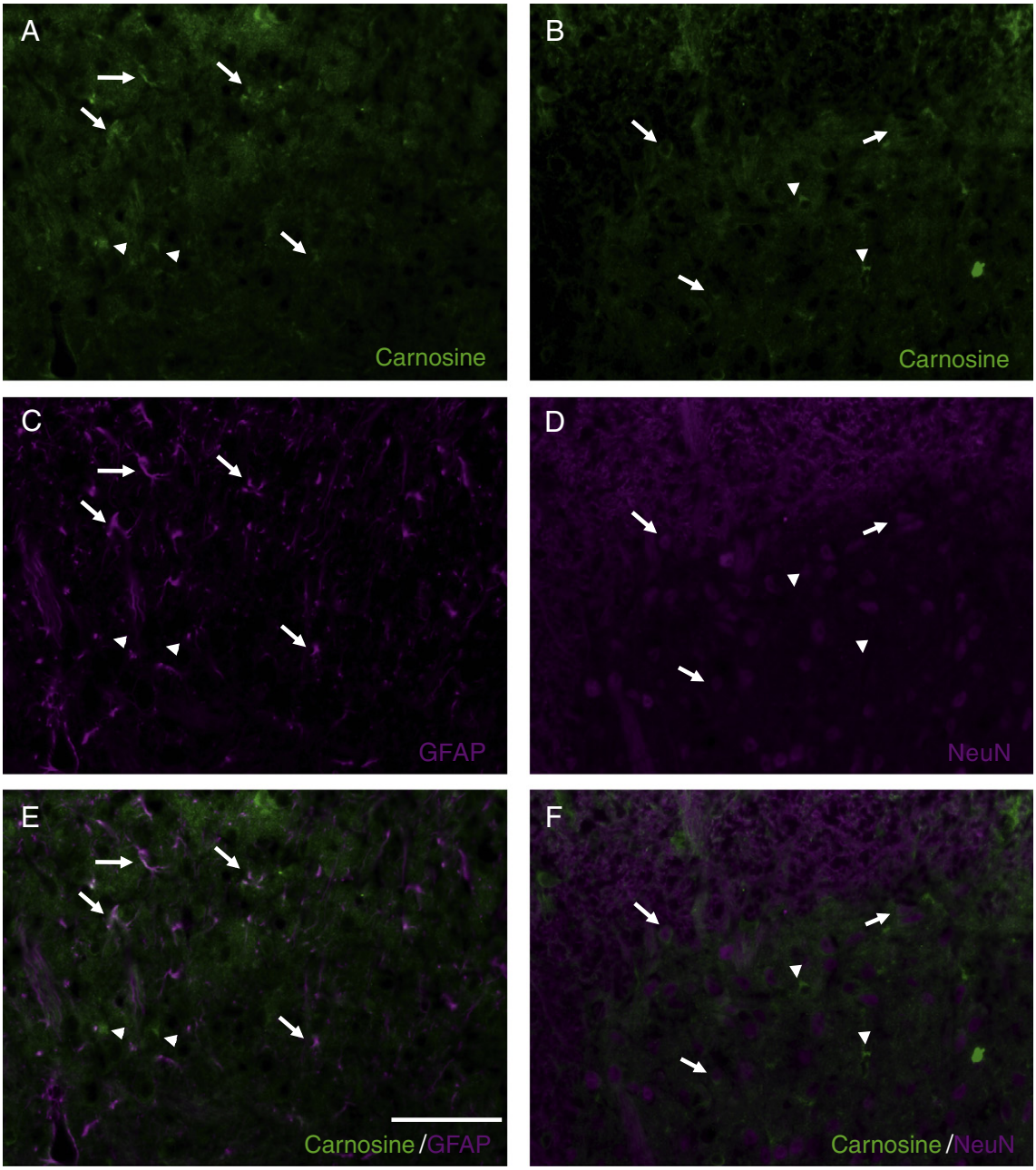

Fig. 2. Carnosine-like immunoreactivity in the spinal cord. In the dorsal horn of the spinal cord, many cells that are immunoreactive to carnosine antisera (A,B) are also GFAP (E)- and NeuN (F)-positive. E and F are merged images of A and C, and B and D, respectively. Arrows indicate carnosine-positive astrocytes (A and E) and neurons (B and F). Arrow heads indicate carnosine-positive cells that were not stained with the respective marker proteins. Scale bars = 50 μm.

then coverslipped with fluorescence mounting medium (Dako, Carpinteria, CA). Fluorescence of immunolabeling was detected using a fluorescence microscope (AxioImager A1; Carl Zeiss, Germany).

2.3. Acetic acid writhing test

After mice were acclimated to individual observation cages for 10 min, they were injected intraperitoneally with 0.6% acetic acid and returned to the individual cages. Abdominal constriction was defined as a mild constriction and elongation that passed caudally along the abdominal wall, accompanied by a slight twisting of the trunk and followed by bilateral extension of the hind limbs. The total number of abdominal constrictions was recorded from 10 to 20 min after acetic acid injection. Carnosine and its vehicle (0.5% carboxymethyl cellulose) were administered orally 1 h prior to the administration of acetic acid.

2.4. Formalin test

The formalin test was performed as described previously (Ohsawa et al., 2008). After mice were acclimated to individual observation cages for 10 min, 25 μl of a 1.5% formalin solution was injected subcutaneously into the dorsal aspect of the right hind paw, and mice were returned to the individual cages. The nociceptive response was recorded for 30 min. Mice licked and bit the injected paw, and these responses were distinct and easily observed. The accumulated response time (secs), i.e. the duration of licking and biting of the injected paw, was measured for each 5-min block.

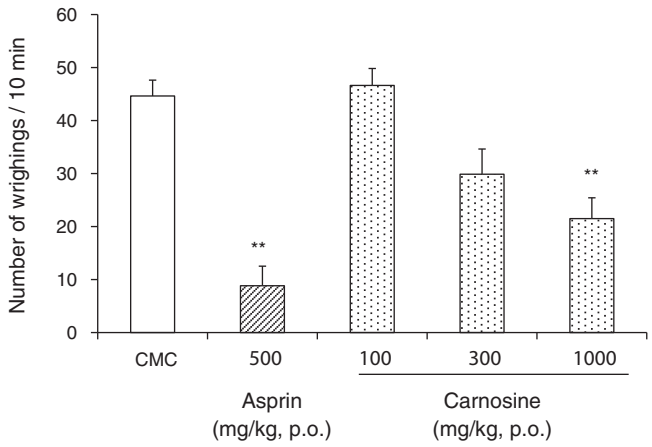

Fig. 3. Effects of p.o. treatment with carnosine on the number of writhing behaviors in mice. Mice were treated with aspirin (500 mg/kg, p.o.) or carnosine (100, 300 and 1000 mg/kg, p.o.) 1 h before the intraperitoneal injection of 0.6% acetic acid. The number of writhings was counted for 10 min after the administration of acetic acid. Data are presented as the mean ± S.E.M. of 10 mice in each group. *P<0.05, **P<0.01 vs. the control.

2.5. Intrathecal administration

Intrathecal injection in a volume of 5 μl was performed a according to the methods of Hylden and Wilcox (1980). The mouse was manually restrained and 30-gauge needle mated to 25 μl Hamilton syringe was inserted between L5 and L6 of the mouse spinal column.

2.6. Drugs

Carnosine was a gift from Hamari Chemicals Ltd. (Osaka, Japan). Other reagents were molecular biology-grade. Carnosine was suspended in a vehicle solution of 0.5% carboxymethyl cellulose for oral administration. When carnosine was administered intrathecally or intraplantarly, it was dissolved in saline (NaCl 0.9%). For oral administration, mice were fasted 24 h before the experiments were started, and carnosine (300, 1000 and 3000 mg/kg) was administered 1 h prior to the injection of 0.7% acetic acid or formalin. Intrathecal (0.1,0.3 and 1 nmol) or intraplantar (1 nmol) administration of carnosine was performed 10 min before the formalin injection. The doses of carnosine were according to the previous report examining the effect of intracerebroventricular administration of carnosine (Kurata et al., 2006) and our preliminary experiment.

2.7. Statistical Analysis

The data are expressed as the mean ± S.E.M. In the behavioral experiment, the statistical significance of differences between groups was assessed with the Newman-Keuls multiple comparison test using GraphPad Prism version 3.0 software. P<0.05 was considered significant.

3. Results

3.1. Expression of carnosine in the spinal cord

Carnosine is found in glial and ependymal cells in the central nervous system (Bonfanti et al., 1999; De Marchis et al., 2000). Since the expression of carnosine in the spinal cord has not yet been reported, we examined the expression of carnosine-immunoreactivity in three vertebrae. The dense expression of carnosine-immunoreactive cell bodies was observed in the dorsal horn of the spinal cord. For example, an abundance of carnosine-immunoreactive cells were present in laminae I to X of the lumbar section, and much fewer carnosine-immunoreactive cells were detected in the deeper laminae (Fig. 1A,B,C). In control experiments, carnosine-immunoreactivity was not detected in any of the spinal sections processed with carnosine-antisera pre-absorbed with the peptide overnight (Fig. 1D). Since the specific cell types that express carnosine are not clear, the spinal sections were double-labeled with antiserum to carnosine and NeuN (a marker for neurons) or GFAP (a marker for astrocytes) (Fig. 2). Some carnosine-immunoreactive cells were GFAP-positive (Fig. 2A,C,E), and others were NeuN-positive (Fig. 2B,D,F).

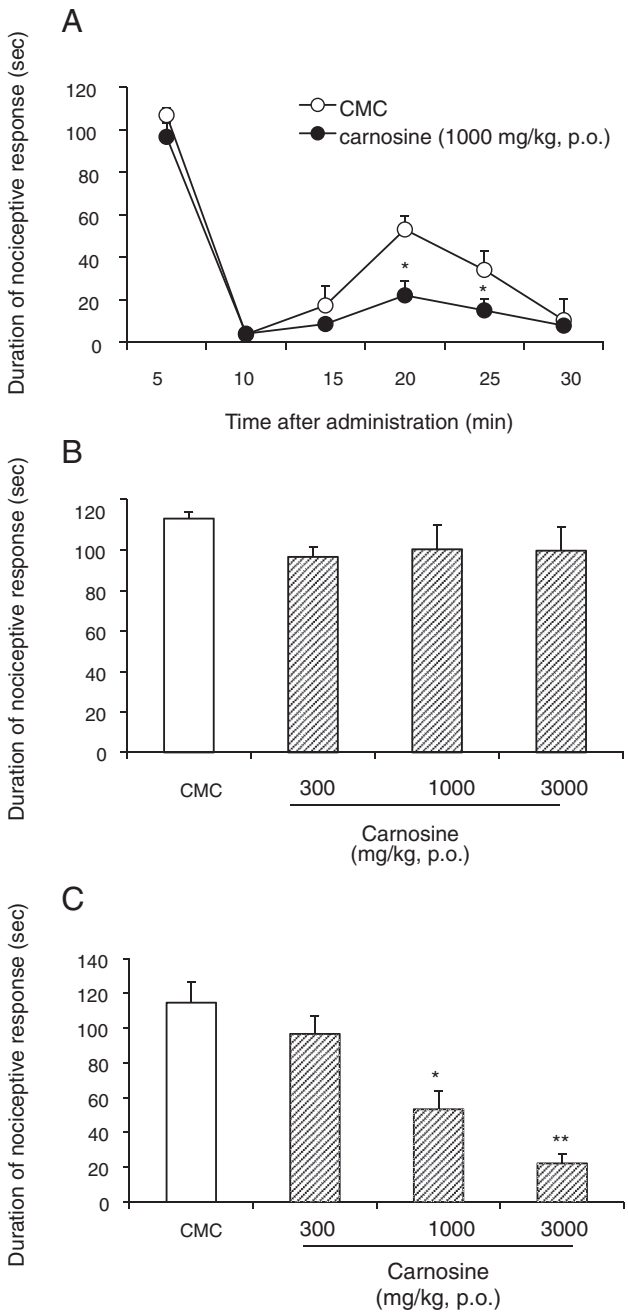

Fig. 4. Effects of p.o. treatment with carnosine on the time course (A) and total duration of the response during the first (B; 0–10 min) and second (C; 10–30 min) phases of the formalin-induced nociceptive response in mice. Carnosine was administered orally 1 h before the subcutaneous injection of formalin. Data are expressed as the total time spent licking and biting the injected paw. Each point and column represent the mean ± S.E.M. of 10 mice in each group. *P<0.05, **P<0.01 compared with the respective vehicle-pretreated group.

3.2. Effect of carnosine on acetic acid-induced writhing

Intraperitoneal injection of 0.6% acetic acid produced an average of 44.6 ± 3.0 abdominal constrictions during the 10-min observation period (Fig. 3). Aspirin significantly reduced the number of acetic acid-

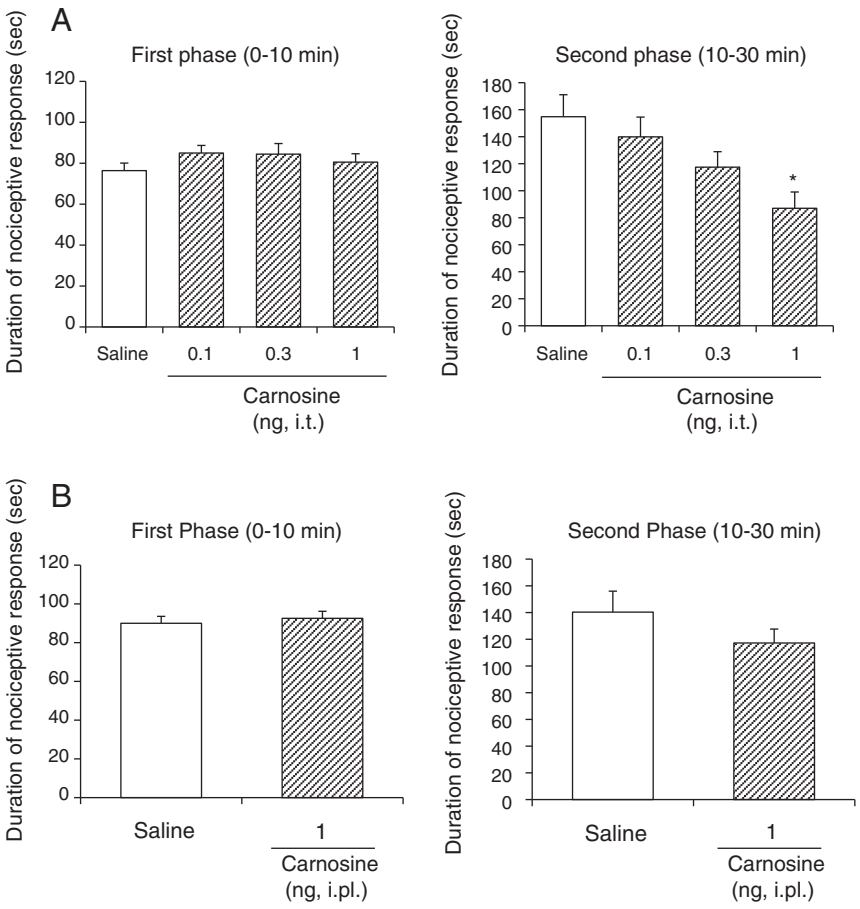

Fig. 5. Effects of intrathecal (i.t.; A) or intraplantar (i.pl.; B) pretreatment with various doses of carnosine on total duration of the response during the first (0–10 min) and second (10–30) phases of the formalin-induced nociceptive response in mice. Carnosine was injected intrathecally or intraplantarly 10 min before the injection of formalin. Data are expressed as the total time spent licking and biting the injected paw. Each column represents the mean ± S.E.M. of 9–10 mice in each group. *$P<0.05$ compared with the saline-treated group.

induced abdominal constrictions (Fig. 3). Oral administration of carnosine decreased the number of writhing behaviors (Fig. 3). This attenuation of acetic acid-induced writhings was statistically significant at doses of 100 and 1000 mg/kg (Fig. 3). The higher dose of carnosine attenuated the number of acetic acid-induced abdominal constrictions to the same extent as aspirin (Fig. 3).

3.3. Effect of carnosine on the formalin-induced nociceptive response

Subcutaneous injection of formalin (25 μl, 1.5%) into the hind paw caused a biphasic response of licking or biting of the injected paw: the first phase started immediately after injection and lasted for about 4 to 5 min, and the second phase began 10 min after injection and lasted about 30 min (Fig. 4A). Oral administration of carnosine did not affect the first phase of the formalin-induced nociceptive response (Fig. 4A, B). In contrast, the second phase of the formalin-induced nociceptive response was dose-dependently attenuated by the oral administration of carnosine (Fig. 4A, C). The attenuation of the second phase of the formalin-induced nociceptive response was statistically significant at doses of 1000 and 3000 mg/kg (Fig. 4C).

3.4. Effect of locally administered carnosine on the formalin-induced nociceptive response

Intrathecal administration of carnosine dose-dependently attenuated the second phase of the formalin-induced nociceptive response (Fig. 5A). The first phase of the formalin-induced nociceptive response was not affected by intrathecal treatment with carnosine (Fig. 5A). The attenuation of the second phase of formalin-induced nociceptive response reached statistically significant at the dose of 1 ng (Fig. 5A). In contrast to intrathecal injection, intraplantar injection of carnosine (1 ng) did not affect any phase of the formalin-induced nociceptive response (Fig. 5B).

4. Discussion

Carnosine has been shown to be expressed in the central nervous system. Our present results indicate that carnosine is expressed in the spinal cord dorsal horn in mice. Recently, carnosine has been shown to be colocalized with GFAP in the cerebral cortex after pMCAO (Rajanikant et al., 2007). We also observed that carnosine was colocalized with GFAP, which indicated that some of the carnosine-positive cells in the spinal cord were astrocytes. Moreover, NeuN-positive cells also expressed carnosine, which indicated that neurons also expressed carnosine. These results prompted us to examine the effect of carnosine on nociception. Treatment with carnosine attenuated the number of acetic acid-induced abdominal constrictions and the second phase of formalin-induced nociception. Moreover, Intrathecal, but not intraplantar, administration of carnosine attenuated the second phase, but not the first phase, of the formalin-induced nociceptive response, indicating that carnosine might partly inhibit spinal sensitization of nociceptive transmission induced by peripheral inflammation. Carnosine has been shown to have several pharmacological actions in neural cells, such as cytosolic buffering, broad antioxidant activity, antiglutamatergic excitotoxicity, and metal ion-chelating effects (Boldyrev et al., 1999, 2004; Bonfanti et al., 1999; Trombley et al., 2000). These actions could modulate spinal nociceptive transmission, since hyperalgesia caused by peripheral inflammation has been

shown to be attenuated by treatment with antioxidant and antiglutamatergic agents (Stanfa and Dickenson, 1999; Zhou et al., 2006). Although the detailed mechanisms of carnosine-induced antinociception are not clear, these broad actions might play a role.

We previously indicated that treatment with zinc carnosine delayed the onset of hypoalgesia in the late stage of diabetes and slightly attenuated the hyperalgesia in the early stage (Kamei et al., 2008). In that study, we confirmed that carnosine is the active substance that underlies the ameliorating effects of zinc carnosine on the changes in the nociceptive threshold in diabetic mice. In the present study, treatment with carnosine attenuated the nociceptive response induced by tissue inflammation. A previous report indicated that peripheral inflammation-induced hyperalgesia is mediated by the production of superoxide both peripherally and centrally (Zhou et al., 2006). Treatment with an antioxidant once the hyperalgesic response was initiated caused a rapid reversal of hyperalgesia (Zhou et al., 2006). Moreover, carnosine reduced the level of reactive oxygen species in mice, which led to neuroprotection in focal cerebral ischemia (Rajanikant et al., 2007). In light of these observations, carnosine might ameliorate spinal oxidative stress induced by peripheral inflammation through its antioxidative properties.

The mechanisms that underlie the carnosine-induced antinociceptive effect in inflammatory pain are not yet clear. The pharmacological effect of carnosine has been shown to be mediated either by carnosine itself or by its metabolite histamine. Recent reports have indicated that a carnosine-histidine-histamine metabolic pathway exists in the central nervous system (Wu et al., 2006). A previous study indicated that the intracerebroventricular administration of histamine attenuates the second phase of the formalin-induced nociceptive response, suggesting that the antinociceptive effect of carnosine is mediated by the production of histamine in the central nervous system. On the other hand, a recent report by Shen et al. (2010) clearly indicated that the effect of carnosine on permanent cerebral ischemia was still observed in histidine decarboxylase knockout mice, and this effect was mediated by the attenuation of glutamate excitotoxicity. This report suggests that carnosine itself directly reduces the increased extracellular content of glutamate after ischemic injury. Since the glutamate content in the spinal cord dorsal horn is increased after peripheral inflammation, carnosine might attenuate this increase in the glutamate level, which would lead to antinociception. Further studies will be needed to identify the putative action site of carnosine in the central nervous system to reveal the mechanisms that underlie the various pharmacological actions of carnosine.

Carnosine at a dose of 1000 mg/kg had almost the same antinociceptive potency as aspirin at a dose of 500 mg/kg. This dose of carnosine appears to be high. The morphine-induced conditioned place preference was attenuated by the intraperitoneal injection of carnosine at doses of 200, 500 and 1000 mg/kg (Gong et al., 2007). Moreover, subcutaneous injection of carnosine at doses of 250, 500 and 750 mg/kg reduced the infarct size in permanent cerebral ischemia (Shen et al., 2010), indicating that the doses of carnosine used in this study are suitable for evaluating its pharmacological action. A human-equivalent dose may be clinically feasible, and the treatment of humans with carnosine is assumed to require chronic treatment. Further studies will be needed to establish the human-equivalent dose and to clarify the exact pharmacological action of this dipeptide.

Taken together, the present results show that L-carnosine has an ameliorative effect on inflammatory pain. Several clinical trials have already shown that L-carnosine is safe. Our present data strongly suggest that L-carnosine may be an important option for the relief of inflammatory or chronic pain.

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