Carnosine Is Neuroprotective Against Permanent Focal Cerebral Ischemia in Mice

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Background and Purpose—Carnosine is a naturally occurring dipeptide with multiple neuroprotective properties. In addition, it is well tolerated in high doses with minimal side effects. The purposes of this study were to determine whether carnosine is neuroprotective in permanent focal cerebral ischemia and to determine potential mechanisms of neuroprotection.

Methods—We investigated the efficacy of carnosine in a mouse model of permanent focal cerebral ischemia. The effects of carnosine were investigated with respect to neuronal damage and infarct formation, endogenous antioxidant status, and matrix metalloproteinase activity.

Results—Carnosine significantly decreased infarct size and neuronal damage when administered at time points both before and after the induction of ischemia. Carnosine also decreased reactive oxygen species levels in the ischemic brain, preserved normal glutathione levels, and decreased matrix metalloproteinase protein levels and activity.

Conclusions—Carnosine is neuroprotective in focal cerebral ischemia and appears to influence deleterious pathological processes that are activated after the onset of ischemia. (Stroke. 2007;38:3023-3031.)

Key Words: brain ischemia • carnosine • neuroprotective agents

In ischemic stroke, effective neuroprotection may require inhibition of multiple deleterious pathological events that are activated by ischemia such as excitotoxicity, oxidative stress, inflammation, acidosis, matrix metalloproteinase (MMP) activation, and blood–brain barrier leakage.1–3 Currently, treatment options for stroke are limited, and many promising experimental drugs have failed in human clinical trials, many due to intolerable side effects.

Carnosine (β-alanyl-L-histidine) is an endogenous dipeptide expressed in many tissues of the body, including the central nervous system, where it is primarily found in glial and ependymal cells.4,5 It is safe and well tolerated and commonly used as a dietary supplement. In vitro and in vivo studies have revealed that carnosine can exert neuroprotective effects through various mechanisms such as cytosolic buffering capabilities, broad antioxidant activity, antiglutamatergic excitotoxicity, and metal ion-chelating properties.5–8 Therefore, carnosine, through its diverse pharmacological activities, could have potential neuroprotective benefits against cerebral ischemia.

Carnosine has been shown to protect neuronal cultures from oxygen–glucose deprivation, an in vitro model of stroke, and to decrease mortality, improve functional ability, and reduce the levels of biochemical markers of damage after global ischemia in rats and gerbils.9–12 Human stroke typically does not involve disruption of blood flow to the entire brain, but rather to a specific region supplied by a single occluded blood vessel, often the middle cerebral artery. Focal ischemia models like the permanent occlusion of the middle cerebral artery (pMCAO) are therefore a better representation of naturally occurring stroke.

In this study, we investigated for the first time the effect of carnosine in mice subjected to pMCAO. Carnosine was evaluated for its effects on infarct size, tissue damage, antioxidant status of the brain, and MMP activation.

Materials and Methods

Focal Cerebral Ischemia

All animal procedures used were approved by the All-University Committee on Animal Use and Care. Focal ischemia was induced by pMCAO in male C57BL/6 mice (22 to 27 g; Charles River Breeding Laboratories, Wilmington, Mass) as previously described.13 Mice were kept under isoflurane anesthesia during the entire procedure and scalp and body temperature were maintained at 37°C by a homeothermic blanket system connected to temperature probes. A 1-cm skin incision was made to create a small subtemporal craniotomy to expose the middle cerebral artery. The artery was occluded using a bipolar coagulator. A Perimed PF-3 laser Doppler perfusion monitor was used to measure blood flow during the surgery and to ensure completeness of occlusion. Measurements were made with the probe placed directly over the middle cerebral artery at a location immediately downstream of the site of occlusion, and the relative change in perfusion units was calculated. The mean reduction in blood flow after occlusion was 92.2%. Animals with less than 80%
reduction in cerebral blood flow were excluded from the study. The incision site was then closed and the animal was allowed to recover from anesthesia. Control mice receiving sham ischemia were subjected to all steps described except for coagulation of the artery. Systolic blood pressure and heart rate (at least 6 separate recordings for each) were measured by a noninvasive tail-cuff apparatus in conscious mice (XBP1000,Kent Scientific). The venous blood pH was measured using an ABL 705 Radiometer.

Carnosine Treatment
L-carnosine (Sigma) was dissolved in 9 g/L sterile saline (100 mg/mL) and administered by intraperitoneal injection. In a first paradigm, saline (n=10) or 100 (n=10), 500 (n=10), and 1000 (n=10) mg carnosine per kilogram were administered 30 minutes before ischemia, and animals were euthanized 24 hours after surgery. In a second paradigm, mice received saline (n=7 for each time point) or 1000 mg carnosine per kilogram (n=7 for each time point) 2 or 4 hours after ischemia supplemented by doses of 500 mg/kg every 6 hours until 24 hours, at which time animals were euthanized. In a third paradigm, sham (n=5 for each treatment) and pMCAO (n=9 for each treatment) mice received saline or 1000 mg/kg carnosine 30 minutes before surgery supplemented by doses of 500 mg/kg every 6 hours until 24 hours, at which time animals were euthanized.

Tissue Collection and Preparation
All animals were euthanized 24 hours after surgery. For 2,3,5-triphenyltetrazolium chloride (TTC) and reactive oxygen species (ROS) stainings, mice were euthanized by an overdose of isoflurane anesthesia followed by cervical dislocation. The brains were then dissected out and used immediately (TTC staining) or kept at -70°C until sectioning (ROS stainings). For ROS staining, 7 serial series of 10-μm thick coronal sections of the entire brain were collected at 350-μm intervals on a Thermelectron cryostat, collected on slides, and either processed immediately or stored at -70°C. For histological and immunohistochemical stainings, animals were transcardially perfused with 30 mL phosphate-buffered saline followed by 50 mL 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Brains were removed, postfixed for 3 hours in the same fixative at 4°C, and cryoprotected in phosphate-buffered 30% methanol. Brains were then transferred to a cryomix containing 15% methanol and 25% glycerol and stored at -70°C. Sections were cut on a Tissue Tek II (Miles) cryostat and collected on slides.

Immunofluorescence Staining
Sections were immunostained as previously described using the following primary antibodies: rabbit anticalcine (1/400; generous gift from Dr Frank L. Margolis, University of Maryland, Baltimore, Md), mouse anti-NeuN (neuronal marker, 1/100; Chemicon), rabbit antiallactylar fibrillary acidic protein (GFAP, glial marker, 1/200; Zymed), and RIR (oligodendrocyte marker, 1/100; monoclonal antibody developed by Dr S. Hockfield and obtained from the Developmental Studies Hybridoma Bank). Primary antibodies were detected using anti-rabbit or anti-mouse secondary antibodies conjugated to Alexa 488 (1/1000; Invitrogen) or Cya nin-3 (1/250; Jackson Immunoresearch). Sections were stained with 10 ng/mL 4,6-diamidino-2-phenylindole for nuclear staining. Control experiments were carried out by replacing the primary antibodies with normal serum, by omitting one immunoreagent of the immunostaining protocol, or by absorption with the corresponding peptide.

Antioxidant Status of the Ischemic Brain
The level of glutathione in the brain was used as a measurement of endogenous antioxidant activity. Total glutathione level was measured using a kit obtained from Sigma-Aldrich following the manufacturer’s protocol. The presence of glutathione was determined by the reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid and detected by measuring an increase in absorbance at 412 nm on a SmartSpec 300 spectrophotometer (Bio-Rad, Hercules, Calif). The levels of reactive oxygen species (ROS) in the brain were measured by an oxidant-sensing fluorescent probe, dihydrofluorescein diacetate, and sections were examined blindly by 3 independent observers and images were captured at 200× magnification by an Olympus BX51 fluorescence microscope. Exposure parameters were primarily adjusted to avoid saturation and kept constant throughout the experiment. For each section with an infarct area (from 7 to 10 depending on the size of the infarct), the total numbers of robustly ROS-positive cells were counted in 2 representative fields of view (core and periphery) of the ipsilateral and contralateral hemispheres. Average background staining of the image analyzed was subtracted. The percentage of ROS-positive cells was determined by counting the ratio of fluorescent cells to 4,6-diamidino-2-phenylindole-positive nuclei.

In Vivo Matrix Metalloproteinase Levels and Activity
The ipsilateral and contralateral cortex from each brain were homogenized on ice in 500 μL buffer (50 mmol/L Tris pH 7.6, 150 mmol/L NaCl, 5 mmol/L CaCl2, 0.5 g/L Brij-35, 10 g/L Triton X-100). Samples were centrifuged 5 minutes at 12 000 g and the supernatant protein concentration measured using the DC Protein Assay (Bio-Rad). The level of reactive oxygen species (ROS) in the brain was measured using an oxidant-sensing fluorescent probe, dihydrofluorescein diacetate, and sections were examined blindly by 3 independent observers and images were captured at 200× magnification by an Olympus BX51 fluorescence microscope. Exposure parameters were primarily adjusted to avoid saturation and kept constant throughout the experiment. For each section with an infarct area, the total numbers of robustly ROS-positive cells were counted in 2 representative fields of view (core and periphery) of the ipsilateral and contralateral hemispheres. Average background staining of the image analyzed was subtracted. The percentage of ROS-positive cells was determined by counting the ratio of fluorescent cells to 4,6-diamidino-2-phenylindole-positive nuclei.

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For gelatin zymography, samples were mixed with sodium dodecyl sulfate sample buffer without reducing agent and run on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels containing 1 g/L gelatin. The gels were renatured 30 minutes in 2.5% Triton X-100 and then incubated overnight at 37°C in 50 mmol/L Tris pH 7.4, 200 mmol/L NaCl, 5 mmol/L CaCl₂, and 0.2 g/L Brij-35. The gels were stained 30 minutes in 5 g/L Coomassie Blue R-250, 50% methanol, 10% acetic acid, and destained in 50% methanol, 10% acetic acid. The gels were scanned into a computer for measurement of band optical density.

For Western blotting, samples were mixed with sodium dodecyl sulfate sample buffer containing DTT, denatured 5 minutes at 95°C, then run on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. Membranes were washed in Tris-buffered saline, blocked 1 hour in Tris-buffered saline plus 0.1% Tween-20 (TTBS) plus 50 g/L nonfat milk, washed 3 times in TTBS, and incubated overnight at 4°C in TTBS with 50 g/L bovine serum albumin and 1:5000 MMP-9 antibody (Affinity BioReagents, Golden, Colo). The membranes were then washed 3 times in TTBS, incubated 1 hour in TTBS plus milk, and 1:20 000 horseradish peroxidase-conjugated secondary antibody (Pierce Biotechnology, Rockford, Ill), and then washed 2 times with TTBS and once with Tris-buffered saline. Membranes were incubated in SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) and exposed to x-ray film. Films were scanned into a computer for measurement of band optical density.

Statistical Analysis

The statistical significance between groups was determined on the basis of one-way analysis of variance test followed by Fisher least significant difference post hoc test (SigmaStat 3.0; SYSTAT Software Inc). Statistical significance was defined at \( P \leq 0.05 \). All data are expressed as mean ± SEM.

Results

Effect of Permanent Occlusion of the Middle Cerebral Artery on Carnosine-Like Immunoreactivity in the Brain

Carnosine has been shown to be present in the mammalian adult brain. To determine and characterize the in vivo effects that cerebral ischemia has on the cerebral expression levels of carnosine, we analyzed and compared the distribu-
Figure 2. A, Effect of single-dose carnosine on infarct size in mice at 24 hours post-pMCAO. Top, TTC-stained sections showing reduction in infarct (white) by carnosine treatment. Bottom, Calculated mean infarct volume for the various treatment groups. B, Effect of multiple-dose carnosine treatment on infarct size beginning at 2 or 4 hours post-pMCAO. C, Top, Microscopic analysis of cresyl violet staining in the mouse cortex at 24 hours post-pMCAO. The infarct core (asterisk) and periphery (arrows) are indicated. Right inset illustrates the presence of pyknotic profiles in the ischemic cortex. Bottom, Quantitative image analysis of the infarct area in saline- or carnosine-treated mice at 24 hours post-pMCAO. Histogram values represent means±SEM. An asterisk indicates statistically significant difference between groups.
tation pattern of carnosine-like immunoreactivity (LI) in the brains of sham-operated and pMCAO mice 24 hours after surgery. As shown in Figure 1A, and as previously reported, a low to medium intensity of carnosine-LI was observed in cortical cells exhibiting glial-like cell morphology (inset in Figure 1A). The distribution pattern of carnosine-LI after pMCAO was strikingly different from that observed in shams (Figure 1A). Whereas no carnosine-LI could be observed in the core of the infarct area (asterisk in Figure 1A), a dramatic increase in the number of carnosine-LI cells and/or intensity of cellular immunostaining was clearly observed at the periphery of the infarct area (arrows in Figure 1B), suggesting that carnosine might play a role after focal ischemia.

**Effect of Carnosine on Physiological Variables After Permanent Occlusion of the Middle Cerebral Artery**

To ensure that the treatment regimens to be used in this study had no negative physiological effects, basal physiological parameters were examined in mice pretreated with 1000 mg/kg carnosine or saline vehicle 30 minutes before pMCAO. Neither ischemia nor carnosine administration had any effect on blood pressure, heart rate, core body temperature, Po2, Pco2, or pH when examined either before or 1 or 3 hours after pMCAO (Figure 1B).

**Carnosine Treatment Reduces Infarct Size After Permanent Occlusion of the Middle Cerebral Artery**

Because carnosine has previously been shown to cross the blood–brain barrier, a new study was conducted to determine whether exogenously administered carnosine could reduce infarct size after pMCAO. As assessed by TTC staining at 24 hour post-pMCAO, carnosine treatment reduced the infarct volume in a dose-dependent manner (Figure 2). Five hundred and 1000 mg/kg carnosine pretreatment significantly reduced the infarct size by 42% (8.09 ± 1.48; P < 0.02) and 49.3% (7.08 ± 1.80; P < 0.01), respectively, when compared with saline vehicle (13.96 ± 1.67) (Figure 2A). To determine whether posttreatment with carnosine is also protective, the effect of 1000 mg/kg carnosine administered 30 minutes after the induction of pMCAO was examined. Posttreatment with carnosine also produced a significant reduction in infarct size (38.3%; 8.62 ± 1.40; P < 0.03; Figure 2A).

Because patients with stroke typically arrive at the hospital hours after a stroke has occurred, the effect of delayed administration of carnosine in multiple doses was also investigated. Ischemic mice received saline or an initial dose of 1000 mg/kg carnosine after either 2 or 4 hours of ischemia supplemented with doses of 500 mg/kg every 6 hours until 24 hours, at which time the animals were euthanized. A statistically significant decrease in infarct volume was observed in animals that received carnosine at 2 hours post-pMCAO (36%; 10.12 ± 1.30, P < 0.01 versus 15.83 ± 1.48; Figure 2B) but not at 4 hours (12.73 ± 1.98 versus 17.18 ± 1.22), indicating the time window during which carnosine must be administered to be effective.

For subsequent experiments, a multiple dosing strategy involving an initial dose 30 minutes before ischemia and additional doses 6, 12, 18, and 24 hours after ischemia was used. Efficacy of this treatment regimen on reducing the infarct size after pMCAO was assessed by cresyl violet staining. The infarct area was readily visible as a lightly stained, well-defined cortical area characterized by the presence of shrunken, pyknotic, and unhealthy cells, thereby indicating the focal nature of the lesion. A statistically significant decrease in infarct size after carnosine treatment was observed in comparison to saline vehicle (50%; 14.75 ± 4.04, P < 0.01 versus 29.46 ± 3.19; Figure 2C).

**Carnosine-Like Immunoreactivity Is Increased in Carnosine-Treated Permanent Occlusion of the Middle Cerebral Artery Mice**

To determine whether carnosine administration had any effects on the distribution pattern of carnosine-LI in the brain, saline- or carnosine-treated mice were analyzed 24 hours after pMCAO. Carnosine treatment markedly increased carnosine-LI throughout the brain, specifically on the side ipsilateral to the injury (Figure 3A). Double immunostainings with neuronal markers (NeuN) and glial markers (GFAP, RIP) indicated that a large number of carnosine-LI cells were also positive for GFAP (Figure 3B), demonstrating their astrocytic phenotype.

**Carnosine Treatment Reduces Oxidative Stress After Permanent Occlusion of the Middle Cerebral Artery**

Several studies have demonstrated significant antioxidant and free radical scavenging properties of carnosine in the brain. Because oxidative stress is a hallmark of stroke-induced neuronal injury, we then determined the effects of exogenously administered carnosine on the oxidative status of the brain 24 hours after pMCAO. Oxidative status was first assessed by determining levels of the endogenous antioxidant glutathione. As shown in Figure 4A, cortical levels of glutathione were significantly decreased 24 hours after pMCAO on the side ipsilateral to the injury (ipsilateral: 1.29 ± 0.08 versus contralateral: 2.17 ± 0.04, P < 0.001). In contrast, carnosine treatment maintained glutathione levels in the ipsilateral cortex at control levels (ipsilateral: 1.91 ± 0.17 versus contralateral: 2.29 ± 0.10, P > 0.05). The antioxidant activity of exogenously administered carnosine was further explored using the dye dihydrofluorescein diacetate, which reacts with ROS to produce a fluorescent product. In saline-treated pMCAO mice, a large number of cells intensely labeled for ROS were observed contralaterally (44.00 ± 5.46) and ipsilaterally (71.1 ± 3.50; Figure 4B and 4C). Carnosine treatment significantly reduced the number of cells with robust ROS staining both contralaterally (22.8 ± 4.47; P < 0.004) and ipsilaterally (54.5 ± 5.65; P < 0.02).

**Carnosine Treatment Inhibits the Increase in Matrix Metalloproteinases After Permanent Occlusion of the Middle Cerebral Artery**

The gelatinases MMP-2 and MMP-9 are induced in the brain after ischemia and are involved in disruption of the blood–brain barrier among other deleterious processes. To determine whether carnosine treatment could inhibit MMP activity in the
brain after ischemia, gelatin zymography was performed on tissue lysates that had been partially purified for gelatin-binding proteins. Compared with sham animals, MMP-9 levels were significantly increased in the ipsilateral cortex after 24 hours of ischemia, but MMP-2 levels were unchanged (Figure 5A). Carnosine treatment significantly inhibited the increase in MMP-9 after induction of pMCAO (1.48 ± 0.38 versus 8.98 ± 2.15; \( P < 0.001 \); Figure 5A). Because the pronounced increase in MMP-9 detected by zymography could be due to both increased activation and increased synthesis of MMPs, the levels of MMP-9 protein in the brain were determined by Western blotting. Levels of MMP-9 protein were significantly increased in the ipsilateral cortex after ischemia, and carnosine treatment inhibited this increase (Figure 5B).

To confirm that carnosine inhibits MMP activity, an in vitro activity assay using recombinant MMP-2 and MMP-9 was performed. The MMPs were incubated with various concentrations (20, 40, 60 mg/mL) of carnosine, and the effect on enzyme activity was determined by the ability to degrade fluorescently labeled gelatin. Carnosine significantly decreased the gelatinase activity of both MMP-2 and MMP-9 in a dose-dependent manner (Figure 5C).
Figure 4. Effect of carnosine on the oxidative status of the brain. A, Glutathione levels after 24 hours of pMCAO. Asterisks indicate statistically significant differences versus contralateral hemisphere. B, Analysis of ROS staining in the cortex from saline- or carnosine-treated mice at 24 hours post-pMCAO. C, Quantitative image analysis of the percentage of ROS-positive cells/field in the infarct area (core) in saline- or carnosine-treated mice 24 hours after pMCAO. Histogram values represent means ± SEM. Statistically significant differences are indicated by an asterisk (*; contralateral versus ipsilateral hemisphere), plus (+; saline versus carnosine, contralateral hemisphere), or number symbol (#; saline versus carnosine, ipsilateral hemisphere).
Discussion

Considerable data suggest that carnosine may favorably influence deleterious pathways that are activated during stroke. Although carnosine has been shown to improve neurological outcomes and decrease mortality in animal models of global cerebral ischemia,\textsuperscript{10–12} it had not been previously tested in focal ischemia models, which more closely resemble stroke. We show for the first time that pre- and posttreatment of mice with carnosine significantly reduced infarct size and neuronal damage after focal ischemia.

We also found that carnosine-LI in the brain was significantly increased after pMCAO. Interestingly, we observed a greater increase in carnosine-LI after carnosine treatment. Because carnosine has been shown to cross the blood–brain barrier,\textsuperscript{16} this increase might be due to entry in the brain of carnosine or its metabolites.\textsuperscript{19} Future studies may use radiolabeled carnosine to confirm if exogenously administered carnosine enters the brain. In our animal model, most of the carnosine-LI was colocalized with GFAP, indicating that most of carnosine-positive cells were astrocytes, corroborating previous reports.\textsuperscript{4} The increase in carnosine immunoreactivity in glial cells and/or increase in the number of carnosine-LI glial cells after ischemia and carnosine administration is interesting because glial cells have many functions that may help to protect neurons under stressful conditions.\textsuperscript{4}

A high rate of oxidative metabolism is a hallmark of the brain. Because free radicals mediate much of the ischemia-induced damage in the brain,\textsuperscript{1,3} and because carnosine is a potential endogenous antioxidant, we analyzed its effects on markers of the oxidative stress pathway. Our results show that
carnosine not only preserved brain levels of the reduced form of glutathione, a crucial endogenous antioxidant, but also significantly reduced the number of ROS-positive cells in the infarct area. Preservation of glutathione levels by carnosine might be due to a reduced oxidation of glutathione in response to the carnosine-induced decrease in free radicals or to an increased expression of its synthesizing enzymes. As a whole, these data suggest that the neuroprotective effects of carnosine after focal ischemia may be mediated in part through its known antioxidant properties.

MMPs have been shown to be involved in degradation of the extracellular matrix, disrupting the blood–brain barrier and allowing inflammatory cells to enter the brain.\textsuperscript{17,18} This study presents the first evidence that carnosine treatment decreased MMP-9 activity and protein levels in the brain after ischemia. In contrast, and confirming previous data,\textsuperscript{20} no change in MMP-2 activity was observed. Further studies are needed to examine the effect of carnosine at other time points after ischemia when MMP-2 protein levels are typically increased, because carnosine was also found to directly inhibit the activity of both MMP-9 and MMP-2 in vitro. Because carnosine is a metal chelator, one possible mechanism of this inhibition may be due to the chelation of the calcium or catalytic zinc required for MMP activity. In addition, inhibition of MMP activity might be due to the reduction of ROS levels by carnosine treatment, because ROS has been shown to be involved in MMP activation.\textsuperscript{18} Whether the neuroprotective effects of carnosine are a direct consequence of ROS and/or MMP inhibition is being investigated in ongoing studies.

The doses of 500 mg/kg and 1000 mg/kg of carnosine needed for neuroprotection in our model appear to be high. However, the human-equivalent doses are lower and may be clinically feasible, although further studies would be needed to establish this.\textsuperscript{21} In addition, the human-equivalent dose may be even lower if the intravenous route of administration is used instead of intraperitoneal administration like in this study.

In conclusion, this study shows for the first time that carnosine is neuroprotective in permanent focal cerebral ischemia. We also show that carnosine may offer pluripotent beneficial effects by decreasing oxidative stress and by regulating MMP activity, further indicating its neuroprotective potential against ischemic injury. Because carnosine is safe, nontoxic, and has the potential to inhibit multiple mechanisms of injury after ischemia, it may be more effective than alternative candidate treatments that block only a single pathway. Future studies aimed at investigating the pharmacokinetics, the optimum dose, effects on behavioral and functional outcome, and the extent of delayed neuroprotection will be helpful to further assess carnosine as a therapeutic option for patients with stroke.

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Disclosures

None.

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