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 Thermoelectron 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% sucrose. Six serial series of free-floating coronal 40-μm thick sections were cut on a Tissue Tek II (Miles) cryostat and collected and stored at −20°C in a cryoprotectant composed of glycerol, ethylene glycol, and phosphate-buffered saline. The first series of sections was mounted on slides and processed for cresyl violet staining. Immunohistochemical and histochemical stainings were performed on selected remaining series.

TTC Staining and Infarct Size Quantification
TTC staining was performed as previously described. Computer images of the stained slices were generated using an HP Scanjet 4470c scanner, and the infarct area was measured using the National Institutes of Health Image/J analysis software (version 1.37). The infarct volume in each slice was calculated by taking the average of the infarct area on both sides of the slice and multiplying it by the section thickness. The infarct volumes from individual sections were then summed to determine the total brain infarct volume and adjusted for edema.

Cresyl Violet Staining and Infarct Size Quantification
Cresyl violet-stained sections were used to assess infarct size. Images of the stained sections were visualized and captured at 100× magnification using a digital camera attached to a Nikon Eclipse TE2000S microscope. The infarct area was measured in each section using SPOT analysis software version 3.5.8 (Diagnostic Instruments Inc.). The borders (periphery) of the infarct area were delineated as the edge between healthy and lesioned tissue. The volume of infarct in each section was calculated by taking the average of the infarct area and multiplying it by the section thickness (240 μm). The infarct volumes from individual sections were then summed to determine the total brain infarct volume.

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). Crude protein lysates were partially purified for gelatin-binding proteins by incubation of 700 μg protein in 200 μL buffer for 1 hour at 4°C with 20 μL gelatin–Sepharose 4B beads (Pharmacia, Uppsala, Sweden). The beads were pelleted by brief centrifugation, washed with buffer, and pelleted again. Bound proteins were eluted for 30 minutes in 60 μL buffer containing 10% dimethyl sulfoxide. Aliquots of purified protein equivalent to 70 μg initial protein were used for zymography and Western blotting.
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 5% 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*≤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.
Carnosine treatment reduces infarct size after pMCAO (Figure 1B). To determine whether carnosine treatment could inhibit MMP activity in the brain, an initial dose 30 minutes before ischemia and are involved in disruption of the blood–brain barrier among other deleterious processes.17,18 To determine whether carnosine treatment could inhibit MMP activity in the brain, several studies have demonstrated significant antioxidant and free radical scavenging properties of carnosine in the brain.5,7 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 ± 0.04; 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 (50%; 14.75 ± 4.04, P<0.01 versus 29.46 ± 3.19; Figure 2C).

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.17,18 To determine whether carnosine treatment could inhibit MMP activity in the brain, we sought 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 treatment reduces infarct size after pMCAO (Figure 1B). 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.5,7 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

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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 3. A, Microscopic analysis of carnosine-LI in the cortex of saline or carnosine treated mice at 24 hours post-pMCAO. The infarct core (asterisk) and periphery (arrows) are indicated. B, Confocal microscopic analysis of carnosine- and GFAP-LI in the cortex of carnosine-treated mice at 24 hours post-pMCAO. Merged picture illustrates the association of carnosine (green) with the glial marker GFAP (red) in the lesioned cortex. Arrows indicate the presence of numerous double-labeled cells (green + red = orange-yellow). Nuclei are stained with 4,6-diamidino-2-phenylindole (blue).
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).
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, 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, this increase might be due to entry in the brain of carnosine or its metabolites. Future studies may use radio-labeled 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. 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.

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, and because carnosine is a potential endogenous antioxidant, we analyzed its effects on markers of the oxidative stress pathway. Our results show that

Figure 5. Effect of carnosine on the synthesis and activity of MMPs. A, Top, Representative gelatin zymography image showing MMP levels in the ipsilateral and contralateral cortex after ischemia or sham surgery. Bottom, Quantitation of gelatin zymography results. *P<0.05 versus pMCAO + saline group. B, Top, Representative Western blot showing MMP-9 levels in the ipsilateral and contralateral cortex after ischemia. MMP-9 was undetectable in sham-treated animals. Bottom, Quantitation of Western blotting results. *P<0.05 versus pMCAO + saline group. C, Effect of carnosine on the activity of recombinant MMPs in vitro. *P<0.05 versus control; †P<0.05 versus 20 mg/mL; ‡P<0.05 versus 40 mg/mL; §P<0.05 versus 60 mg/mL.
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.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,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.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.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.

References
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