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Modulation of mitochondrial function and autophagy mediates carnosine neuroprotection against ischemic brain damage

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Abstract

Background and Purpose—Despite the rapidly increasing global burden of ischemic stroke, no therapeutic options for neuroprotection against stroke currently exist. Recent studies have shown that autophagy plays a key role in ischemic neuronal death and treatments that target autophagy may represent a novel strategy in neuroprotection. We investigated whether autophagy is regulated by carnosine, an endogenous pleiotropic dipeptide which has robust neuroprotective activity against ischemic brain damage.

Methods—We examined the effect of carnosine on mitochondrial dysfunction and autophagic processes in rat focal ischemia and in neuronal cultures.

Results—Autophagic pathways such as reduction of phosphorylated mTOR/p70S6K and the conversion of LC3-I to LC3-II were enhanced in the ischemic brain. However, treatment with carnosine significantly attenuated autophagic signaling in the ischemic brain, with improvement of brain mitochondrial function and mitophagy signaling. The protective effect of carnosine against autophagy was also confirmed in primary cortical neurons.

Conclusion—Taken together, our data suggest that the neuroprotective effect of carnosine is at least partially mediated by mitochondrial protection, and attenuation of deleterious autophagic processes. Our findings shed new light on the mechanistic pathways that this exciting neuroprotective agent influences.

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Introduction

Despite the high prevalence and the increasing global burden of ischemic stroke, there are no approved neuroprotective agents in clinical use. The only approved therapy is thrombolysis with tissue plasminogen activator (tPA), which has a narrow therapeutic window and hemorrhagic side effects that limit clinical use. There have been extensive efforts to develop novel therapeutic candidates for ischemic stroke.^{1,2} However, numerous promising candidates have failed in clinical trials due to a number of factors which include poor preclinical study design, illogical clinical translation of preclinical data, poor efficacy and serious side effects.^{3,4} Moreover, understanding the precise mechanisms through which candidate agents exert their protective effects is an important and critical part of therapy development. Agents that influence multiple deleterious pathways are more likely to be efficacious clinically.^{5,6}

There is increasing evidence that autophagy, a highly regulated cellular process that involves degradation of cellular proteins and organelles, can contribute to neuronal death during brain ischemia. Enhancement of autophagic processes was observed in brain after hypoxic-ischemia,⁷ and the occurrence of autophagy measured by conversion of LC3-I to LC3-II during brain ischemia has been confirmed by *in vivo* imaging.⁸ Although controversy exists whether autophagy contributes to cell death or cell survival,⁹⁻¹¹ recent observations using inhibitors or modulators of autophagy revealed that autophagy mediates neuronal cell death during ischemia.^{12,13} Wen et al¹⁴ observed autophagy in focal cerebral ischemia, and demonstrated that treatment with inhibitors of autophagy significantly reduced brain damage. Data also exist showing that neuronal death during ischemia is mediated by oxidative stress generated from autophagosomes and mitochondria that are participating in the autophagic process.¹⁵

Activation of autophagic pathways is associated with perturbations in mitochondrial function.¹⁶ Mitochondrial damage is known to result in activation of mitophagy, a specific type of autophagy that eliminates dysfunctional mitochondria,^{17,18} under normal as well as pathological conditions including cerebral ischemia.¹⁹ Despite the increasing attention on autophagy as a novel target for stroke therapy development, studies on agents that modulate autophagy and that could be used clinically are still limited.

Carnosine, an endogenous dipeptide, is a pleotropic agent that exhibits diverse activities including anti-oxidant, anti-matrix metalloproteinase, heavy metal chelating and anti-excitotoxic properties.^{20,21} We recently showed that carnosine robustly reduced brain damage after ischemic stroke.²²⁻²⁵ Post-treatment with carnosine protected against histological brain damage both in permanent- and transient-ischemic rat models with a wide clinically relevant therapeutic window of 9 hr and 6 hr, respectively, along with improvements in functional outcomes.²³ Carnosine did not exhibit any side effects or organ toxicity.^{23,25} Along with our observation, others have also reported the robust

neuroprotective activity of carnosine.²⁶⁻²⁸ However, it is not known whether carnosine can influence autophagy in the ischemic brain.

In the current study, we have investigated whether carnosine has the ability to modulate autophagic processes in the ischemic brain using both *in vitro* and *in vivo* approaches. We extended our studies to mitochondria and showed that carnosine has a significant and profound effect on autophagy and associated mitochondrial perturbations that occur during ischemia. Our findings support the pleiotropic multimodal action of carnosine and provide, for the first time, proof of its influence on autophagy.

Materials and Methods

More details are provided in the online supplemental material.

Animals

All animal experiments were conducted using adult male Sprague-Dawley rats weighing 250 to 300 g (Harlan, Koatech, Korea) and performed in accordance with the NIH Policy and Animal Welfare Act under the approval by Institutional Animal Care and Use Committee (IACUC) at Hanyang University.

Blinding and Randomization

Treatment groups were allocated in a randomized fashion. Investigators were blind to the allocation of treatment during surgeries and outcome evaluations.

Treatments

Carnosine was obtained from Sigma and dissolved in saline. Carnosine (1,000 mg/kg) was administered into the lateral tail vein at 6 hr after ischemic onset both in permanent and transient models. The choice of this dose and time window is based on previous dose finding studies.²²⁻²⁵

Ischemic stroke in rats

Permanent or transient focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO).²³ Ischemia was initiated by a silicone-coated 4-0 monofilament nylon suture (Doccol Co.) as described previously.^{23,29}

Calculation of infarct volume

At 24 hr after onset of ischemia, rats were euthanized by isoflurane overdose, and the isolated brains were cut into 2 mm sections. The infarct volume for each section was calculated by 2% triphenyltetrazolium chloride (TTC).³⁰

Assessment of neurological function

Deficit in neurological function was evaluated by behavioral tests including the adhesive tape removal test and a Rota Rod test, at 24 hr after tMCAO (6 hr ischemia).^{23,31} All rats were trained to the tests for 5 consecutive days before focal ischemia.

Brain homogenization and mitochondria isolation

Brain samples between bregma levels +2 and -4 mm, which include ischemic core and penumbra, were rapidly isolated at 24 hrs after MCAO, and brain homogenates were obtained by homogenization in isolation buffer. Brain mitochondria was further isolated using Percoll gradient centrifugation.³²

Western Blot of brain homogenate or isolated brain mitochondria

Processed brain homogenates or brain mitochondria were examined in western blot using Tris-HCl SDS-PAGE.^{23,32} Detailed information on primary antibodies is described in the online supplemental material.

Complex I activity

Complex I activity in isolated brain mitochondria was measured using colorimetric method as previously described with 2,6 dichloroindophenol (DCIP).³³

In vitro culture of primary cortical neurons

Primary cortical neuronal cultures were established as described previously.²² Neuronal cultures were maintained in a CO₂ incubator at 37°C, and used between days *in vitro* (DIV) 7 and 11.

NMDA-induced excitotoxicity

Ischemic neuronal damage was examined by N-methyl-daspartate (NMDA)-induced excitotoxicity.³⁴ NMDA-induced cytotoxicity was measured at 24 hr after NMDA exposure by leakage of lactate dehydrogenase (LDH). Alterations in cellular proteins were assessed by western blot as described earlier, with cell lysates extracted from neuronal cells using RIPA buffer (Thermo Scientific). To examine carnosine protection, cells were pretreated with carnosine for 30 min prior to NMDA stimulation.

Statistics

We calculated the means and standard errors of means (SEM) for all treatment groups. Differences in values were analyzed using Student t-test or analysis of variance (ANOVA), as appropriate, using SPSS software (Chicago, IL). Multiple comparisons were made using one-way ANOVA followed by Tukey test. Two-tailed Student's t-test analysis was used for comparing values between two groups. In all cases, a p value of < 0.05 was considered significant.

Results

Carnosine protects the ischemic brain in focal stroke

First, we examined the neuroprotective effect of carnosine in rat focal ischemia. All physiological variables including body temperature and cerebral blood flow (CBF) were maintained in the reference range. Induction of focal ischemia was attained by middle cerebral artery occlusion (MCAO) and verified by monitoring of CBF. Post-treatment with carnosine (1000 mg/kg) at 6 hr significantly reduced brain infarct volume (Fig. 1A),

measured by TTC-staining. Similarly, we found that carnosine improved functional outcomes following 6 hr transient MCAO, using a variety of tests which included the latency for removal of adhesive tape placed on forelimbs and the latencies to fall off from the accelerating Rota Rod, respectively.^{23,31} (Fig. 1B and 1C).

Carnosine reduced autophagy in brain homogenates

To investigate whether autophagic processes are involved in carnosine mediated protection, we examined the extent of conversion of LC3-I to LC3-II, an important marker of autophagy that is responsible for formation of autophagosome.³⁵ A significant increase in LC3-II formation was observed in the ipsilateral hemisphere following ischemia. However, this increase in LC3-II formation was attenuated by treatment with carnosine (Fig. 2A). It is also well established that inhibition of the mTOR pathway plays a key role in autophagy.³⁶ To investigate the effect of carnosine on the autophagic signaling pathway, we measured the levels of phospho-mTOR (p-mTOR) and phospho-p70S6K (p-p70S6K), a representative downstream target of mTOR,³⁷ in brain homogenates after ischemia. Carnosine did not affect the basal activity of mTOR; similar levels of p-mTOR were observed in hemispheres contralateral to the ischemia in both saline- and carnosine-treated rats (Figure 2B). Ischemia inhibited the phosphorylated levels of mTOR, but this inhibition was blocked by carnosine. Similarly, reductions in the levels of p-p70S6K in ischemic brain were also reversed by carnosine (Fig. 2B). Taken together, these findings support the modulating role of carnosine on autophagy in the ischemic brain. While mTOR-autophagy pathways were significantly influenced by ischemia and reversed by carnosine, the level of phosphorylated ERK 1/2 was not changed either by ischemia or carnosine treatment (Fig. 2B), showing that the modulation of autophagic proteins by carnosine is not a non-specific epi-phenomenon.

Carnosine attenuates ischemic injury to mitochondria

We have previously reported that carnosine reversed the impairment of mitochondrial permeability transition in primary neurons and astrocytes. Since it is well established that mitochondrial dysfunction contributes to autophagy induction,^{16,18} we examined whether carnosine protected against mitochondrial damage and mitophagy. Ischemia resulted in decreased activity of complex I in isolated brain mitochondria suggesting impairment in mitochondrial respiratory function. Ischemic mitochondrial dysfunction was significantly reversed in mitochondria isolated from carnosine-treated rats (Fig. 3A). To determine if there is a link between mitochondrial dysfunction and autophagy, we examined the levels of p-Drp1 and Parkin which play key roles in mitochondrial fragmentation and mitophagy during cell death, respectively.³⁸⁻⁴⁰ The mitochondrial levels of p-Drp1 and Parkin were significantly increased by ischemia, but the increase of p-Drp1 and Parkin were attenuated by carnosine treatment (Fig. 3B).

While the levels of p-Drp1 and Parkin were increased by ischemia, the levels of cytochrome C and apoptosis-inducing factor (AIF) were significantly decreased in brain mitochondria following ischemic insult. Since cytochrome C and AIF are released from mitochondria to the cytosol during mitochondrial damage,^{32,41} these results were consistent with mitochondrial dysfunction. Carnosine potently inhibited the release of AIF and cytochrome C, demonstrating its protective activity on mitochondrial damage (Fig. 3B).

Carnosine protects against neuronal autophagy in culture

Primary cortical neurons were transiently exposed to toxic levels NMDA, and cytotoxicity and autophagic signaling pathways were examined. As shown in Figure 4A, NMDA induced significant cytotoxicity in primary cortical neurons, and NMDA-cytotoxicity was reduced by carnosine treatment. Interestingly, autophagic signaling pathways including LC3-II formation and mTOR de-phosphorylation were significantly enhanced by NMDA exposure, and carnosine reversed these changes (Fig. 4B), confirming the protective effect of carnosine against ischemia-induced neuronal autophagy.

Discussion

Stroke involves a cascade activation of multiple deleterious pathways,^{2,42,43} and therefore a drug candidate that specifically modulates a single pathway is not likely to show clinical efficacy against ischemic brain damage. Many therapeutic candidates including neuroprotectants which had strong protective activity pre-clinically have failed in clinical trials.^{1,4} One major reason for this is that past strategies have focused on targeting one pathway. We have shown that carnosine is an exciting candidate for development as a stroke therapy.^{23,25} It is safe and efficacious with a large clinically relevant therapeutic time window. Moreover, it is a pleiotropic agent that favorably modulates several deleterious pathways that contribute to cell injury and cell death during and after ischemia.^{21,44} We show here, using *in vitro* and *in vivo* approaches that carnosine has a profound and significant effect on autophagy, a recently identified noxious pathway in ischemic stroke. We believe that the current study underlines the translational importance of carnosine as a therapeutic candidate against ischemic stroke where multiple deleterious pathways aggravate neuronal damage.

Autophagy is the cellular process that mediates degradation of cellular proteins and organelles and maintains homeostasis.⁴⁵ Despite its essential role in normal cellular physiology, excessive activation of autophagic pathways is also reported to be highly associated with many disease states including brain damage.^{46,47} Autophagic cell death has been referred to as type II cell death, which is one of the major types of cell death along with apoptotic (type I) and necrotic (type III) cell death.^{48,49} While necrotic and apoptotic cell deaths have long been considered as the main pathological events in ischemic stroke,^{50,51} autophagy has been recently recognized as a possible deleterious event also. Activation of autophagic signaling was observed in ischemic brain,⁵² mediating ischemic neuronal death.¹⁰ Notably, autophagic cell death was found to be the most important contributing pathway in neonatal cerebral ischemia relative to apoptosis and necrosis.⁵³ Autophagy-inhibitors such as 3-MA significantly reverse ischemic brain damage¹⁴ and inhibition of autophagy was suggested to be the main mechanism of ischemic post-conditioning neuroprotection.⁵⁴ Conversely, it has also been reported that autophagy may play a dual role in neuronal survival and death during ischemia,¹⁰ and further studies on the exact molecular targets which switch beneficial autophagy to detrimental autophagy would give valuable insights for development of treatments that modulate autophagy.

The role of mitochondrial dysfunction has been proposed as a contributor to autophagy.¹⁶ We and others have previously shown that ischemic insults to the brain induced

mitochondrial permeability transition (MPT) resulting in damage to mitochondrial function in neurons.^{23,41} Onset of mitochondrial dysfunction is closely linked to initiation of autophagy in I/R injured myocytes,⁴⁶ in rat hepatocytes,⁵⁵ and in neurons.¹⁵ Damaged mitochondria releases cytochrome C (cyt C), AIF, and reactive oxygen species,¹⁷ which promote mitophagy, a form of autophagy that is involved in the removal of dysfunctional mitochondria. Recent data suggests that Parkin, an ubiquitin ligase that mediates mitophagy,⁴⁰ is recruited to the damaged mitochondria.^{36,56} In this report, we observed the increased recruitment of Parkin to the mitochondria, and loss of AIF and cyt C from mitochondria in ischemic brain, which were significantly attenuated by carnosine, demonstrating its protective effect against mitophagy and ultimately autophagic neuronal death. Similarly, Mehta et al⁵⁷ showed that selenium conserved mitochondrial function and stimulated mitochondria biogenesis, along with reduced autophagy in glutamate-induced neuronal toxicity.

Interest in the development of carnosine as an endogenous pleiotropic molecule for therapeutic use clinically has been increasing.^{20,44,58-60} Here we focused on the potential of carnosine against ischemic stroke. Several previous reports showed that carnosine also had beneficial activities in neurodegenerative diseases including Alzheimer diseases,⁶¹ and dementia.⁶² Of note, dysregulation of autophagic processes have been recently recognized to contribute to the progress of these neurodegenerative diseases.^{63,64} Further elucidation of carnosine's effects on autophagy in these neurodegenerative diseases is needed.

In summary, we have demonstrated that carnosine inhibits ischemia-induced autophagy and mitochondrial damage. This novel action of carnosine adds to the other body of compelling data that supports the development of carnosine as a therapeutic agent against ischemic stroke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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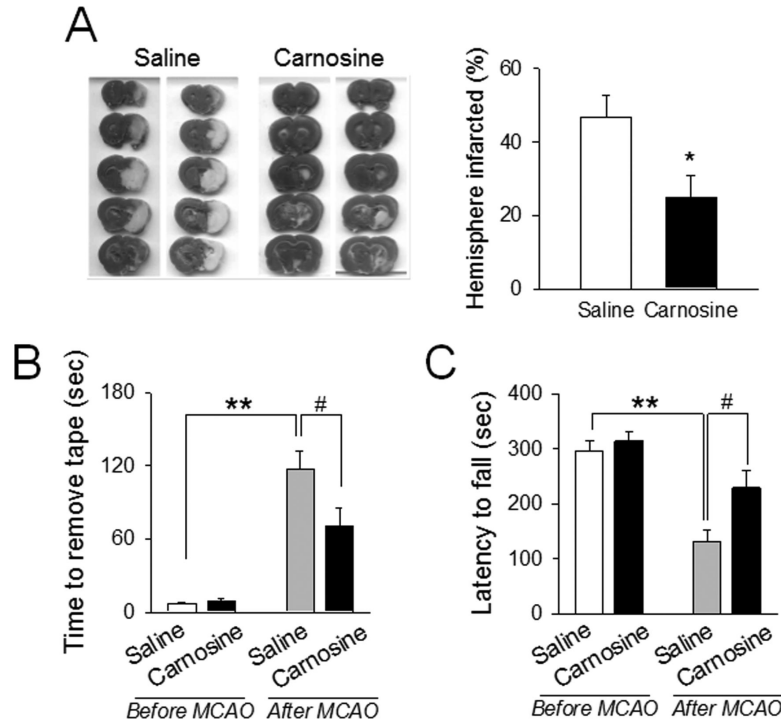


Figure 1. Protective effect of carnosine against brain damage during ischemic stroke
 Ischemic stroke was achieved by middle cerebral artery occlusion (MCAO) in rats. **(A)** Carnosine (1000 mg/kg) was administered 6 hr after onset of ischemia. Infarct volume was determined by 2,3,5-triphenyltetrazolium chloride staining at 24 hr after MCAO. The representative photos are shown. N=13-15. * $p < 0.05$ vs. saline-treated rats. **(B and C)** Carnosine (1000 mg/kg) was administered to rats at 6 hr after ischemic onset during transient MCAO (6 hr ischemia/18 hr reperfusion). Behavioral tests were performed at 24 hr before and after ischemia. **(B)** Somatosensory deficit was determined using the Adhesive Tape tests, where required time to remove adhesives on fore limbs were measured. **(C)** In the RotaRod test, motor-ambulatory function was determined. Latencies to fall off from the rotarod with accelerated speeds were measured. B: N=13-15, C: N=15-16. ** $p < 0.01$, # $p < 0.05$ vs. the corresponding group. Data were expressed as mean \pm SEM and analyzed by Student's t-test.

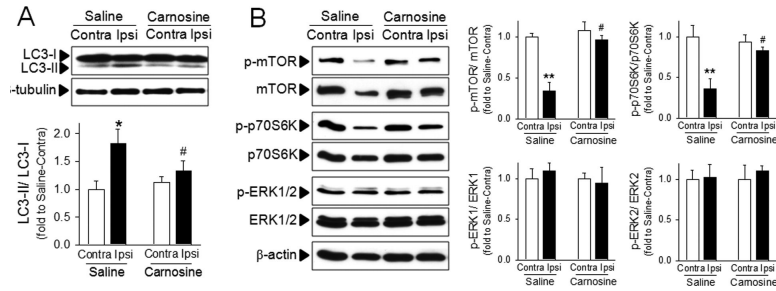


Figure 2. Inhibitory effect of carnosine on autophagy in ischemic brain

Brain homogenates were isolated from contralateral (Contra) or ipsilateral (Ipsi) hemispheres from saline- or carnosine (1000 mg/kg; 6 hr post treatment)-administered rats following pMCAO. **(A)** The extent of autophagy was examined using the conversion of LC3-II from LC3-I. **(B)** Autophagic signaling was examined by phosphorylation of mTOR, p70S6K and ERK. The representative bands are shown. Relative density of each band was analyzed by ImageJ. N=4. * $p < 0.05$, ** $p < 0.01$ vs. contralateral hemisphere from saline-treated rats. # $p < 0.05$ vs. ipsilateral hemisphere from saline-treated rats. Data were expressed as mean \pm SEM and analyzed by Student's t-test.

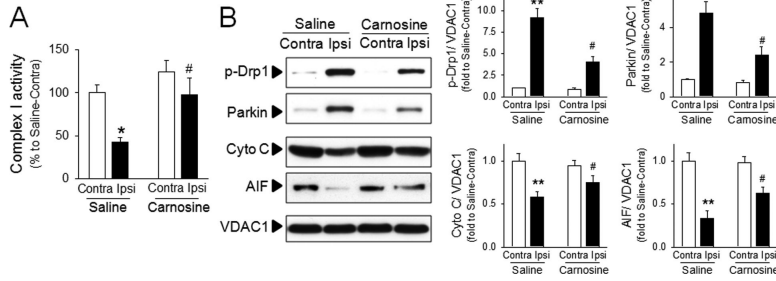


Figure 3. Protective effect of carnosine on mitochondrial damage in ischemic brain
 Brain mitochondria were isolated from contralateral (Contra) or ipsilateral (Ipsi) hemispheres from saline- or carnosine (1000 mg/kg; 6 hr post treatment)-administered rats following pMCAO. **(A)** Complex I activity was measured using colorimetric method. **(B)** The extent of mitochondrial fragmentation and mitophagy was examined using the level of p-Drp 1 and Parkin. Mitochondrial levels of apoptosis inducing factor (AIF) and cytochrome C were measured. Relative density of each band was analyzed by ImageJ. N=4. *p<0.05, **p<0.01 vs. contralateral hemisphere from saline-treated rats. #p<0.05 vs. ipsilateral hemisphere from saline-treated rats. Data were expressed as mean ± SEM and analyzed by Student's t-test.

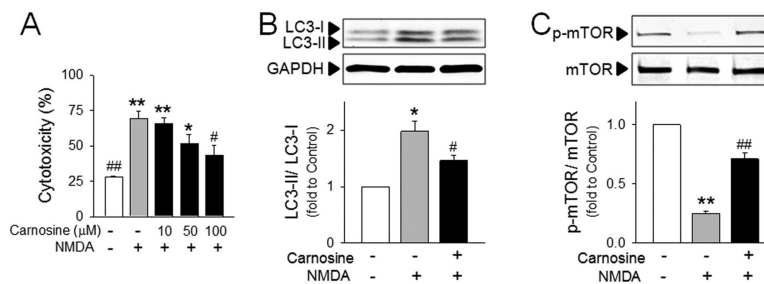


Figure 4. Inhibitory effect of carnosine on neuronal autophagy following NMDA stimulation

Primary cortical neurons were pre-treated with carnosine 30 min prior to exposure to NMDA (N-methyl-d-aspartate; 25 μ M). **(A)** Neuronal cell death was determined by extent of lactate dehydrogenase leakage at 24 hr after NMDA stimulation. N=5. **(B and C)** The conversion of LC3-II from LC3-I **(B)** and the phosphorylation of mTOR **(C)** in NMDA (25 μ M)-stimulated primary neurons with or without carnosine (100 μ M) pretreatment. The representative bands are shown. Relative density of each band was analyzed by ImageJ. N=3. *p<0.05, **p<0.01 vs. control group. #p<0.05 vs. NMDA-treated group. Data were expressed as mean \pm SEM and analyzed by one way ANOVA followed by Tukey test (A) or by Student's t-test (B and C).