Original Article

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Regulation of Mitochondrial Function and Energy Metabolism: A Primary Mechanism of Cytoprotection Provided by Carnosine

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Abstract

Carnosine is a dipeptide abundantly found in different tissues. Several pharmacological properties have been attributed to carnosine. On the other hand, the precise mechanism of cytoprotection provided by carnosine remains obscure. The current study aimed to evaluate the direct effect of different concentrations of carnosine on cellular mitochondria as an essential target involved in cytoprotection or cytotoxicity. Liver mitochondria were isolated and exposed to carnosine (0.01-20 mM). Mitochondrial depolarization, dehydrogenases activity, reactive oxygen species (ROS) formation, mitochondrial swelling and permeability, and ATP content were assessed. On the other hand, the effect of carnosine supplementation on calcium (Ca^{2+}) overload-induced mitochondrial injury was evaluated. It was found that concentrations between 0.01-20 mM of this peptide preserved mitochondrial indices of functionality in a Ca^{2+} overloaded environment. These data represent regulation of mitochondrial function as a primary mechanism for the protective properties of carnosine.

Keywords: Apoptosis; Bioenergetics; Cell death; Cytoprotective; Peptide.

1. Introduction

Carnosine (β-alanyl-L-histidine; CA) is a dipeptide widely investigated for its physiological as well as pharmacological roles (1, 2). CA is present at high concentrations in human skeletal and cardiac muscle, brain, kidney, stomach, and olfactory bulbs (3-5). Several biological roles have been identified for CA and its associated compounds (6-9). Antioxidant and reactive species scavenging properties are essential characteristics of CA which are firmly attributed to the protective properties of this molecule (3, 10-12). CA is an excellent scavenger of reactive oxygen species including singlet oxygen, hydroxyl and superoxide radicals (3, 13, 14). Some investigations mentioned that CA effectively blunts biomembrane disruption both at cellular and organelle level (3, 13, 14). CA is also repeatedly investigated for its protective properties against a wide range of diseases and xenobiotic-induced toxicity (15-20). On the other hand, the direct effect of CA on isolated mitochondria has not been evaluated so far.

Several investigations mentioned the positive effects of CA on mitochondria (21-23). It has been found that CA effectively preserves mitochondrial membrane potential, regulates mitochondrial matrix pH, and enhances mitochondrial defense mechanisms (21-23). Hence, a significant part of cytoprotection provided by CA might be mediated through its effects on cellular mitochondria. On the other hand, the involvement of the mitochondria in regulating cytoplasmic Ca^{2+} level has been well-established (24). Cellular ROS formation and

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oxidative stress, mitochondrial dysfunction, and Ca^{2+} overload are well-known mechanistically interconnected events which finally lead to cell death (24). In the current study, Ca^{2+} was used as a stressor to test the mitochondria-protecting properties of CA.

The current study was designed to evaluate the direct effect of CA on cellular mitochondria and investigate the effect of different concentrations of carnosine on this organelle aimed at understanding the mechanism(s) of CA cytoprotection.

2. Materials and methods

2.1 Chemicals

Carnosine was purchased from Sigma (St. Louis, MO, USA). 4,2 Hydroxyethyl,1-piperazineethanesulfonic acid (HEPES), 3-(N-morpholino) propane sulfonic acid (MOPS), Sucrose, Dimethyl sulfoxide (DMSO), D-mannitol, Bovine serum albumin (BSA; Fatty acid free),Thiobarbituric acid (TBA), 3-[4,5dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Rhodamine 123 (Rh 123), Coomassie brilliant blue, Ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), Hydroxymethyl amino methane hydrochloride (Tris-HCl), Sodium succinate, and Ethylenediaminetetraacetic acid (EDTA) were purchased from Merck (Darmstadt, Germany). All salts (Analytical grade) for preparing buffer solutions were purchased from Merck (Darmstadt, Germany).

2.2. Animals

Male BALB/c mice (20-30 g) were obtained from Animal Breeding Center of Shiraz University of Medical Sciences, Shiraz, Iran. Mice were housed in cages on wood-chip bedding at a temperature of 23±2 °C and relative humidity of ≈40%. Animals had free access to tap water and a standard chow diet (Behparvar®, Tehran, Iran). Mice were handled according to the animal handling protocol approved by thelocal ethics committee at Shiraz University of Medical Sciences, Shiraz, Iran (#15034).

2.3. Liver Mitochondria isolation

Mice liver mitochondria were isolated as previously described (25). Briefly, animals were anesthetized (ketamine/xylazine, 50/10 mg/kg, i.p) and their liver was excised and washed with ice-cold sodium chloride (saline 0.9%) (25, 26). The liver was homogenized in a buffer solution containing 225 mM sucrose, 70 mM mannitol, 0.5 mM EGTA, 0.1% essential fatty acid-free bovine serum albumin (BSA), 2 mM HEPES (pH=7.4), at a 10:1 buffer to liver tissue (v/w) ratio (20, 25). Afterwards, the liver homogenate was centrifuged at 1,000×g for 10 minutes at 4 °C to remove intact cells and nuclei. The supernatants were further centrifuged (15,000×g, 4 °C, 10 minutes) to help precipitate the heavy membrane fractions (mitochondria) (27, 28). This step was repeated four times using fresh buffer medium. As mentioned, all manipulations for liver mitochondria isolation were performed at 4 °C or on ice to minimize mitochondrial injury (25).

2.4. Mitochondrial Swelling assay

Mitochondrial swelling was assessed using the light scattering method (25). For this purpose, the isolated mitochondria were suspended in a buffer containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes-KOH, and pH=7.2 (29, 30). Light absorbance at λ=540 nm was monitored (Constant temperature of 30 °C) with a FLUOstar Omega® multifunctional fluorescent microplate reader (25, 31, 32). It is accepted that a decrease in light absorbance is related to the increase in mitochondrial volume (32). Therefore, as mitochondria are more swelled, the differences between light absorbance of the two time points are higher. The differences between the absorbance of samples at λ=540 nm (ΔOD540 nm) were determined and the results were reported as mitochondrial maximal swelling amplitude (25, 32).

2.5. Mitochondrial membrane potential

The uptake of the cationic fluorescent dye, rhodamine 123 by polarized mitochondria, was used for the estimation of mitochondrial membrane potential (25, 33). For this purpose, the mitochondrial fractions (0.5 mg protein/ml) were incubated with rhodamine 123 (Final concentration of 10 µM) in a buffer solution containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH=7.2 (30

min, 37 ºC, in the dark) (32, 33). Samples were centrifuged (10,000 g, 1 minute, 4 ºC) and the fluorescence intensity of the supernatant was monitored using a FLUOstar Omega® multifunctional fluorescent microplate reader at the $\lambda_{excitation}$=485 and $\lambda_{emission}$=525 nm (25, 34).

2.6. Mitochondrial ATP level

A luciferase-luciferin-based kit (Promega ENLITEN®) was used to determine mitochondrial ATP reservoirs (20, 35). Buffer solutions and mitochondrial samples were prepared based on the kit instructions. Finally, the luminescence intensity of samples was assessed at λ=560 nm using a FLUOstar Omega® multifunctional microplate reader (36). For standardization of the data, the protein concentrations of samples were determined by the Bradford method (37).

2.7. Mitochondrial dehydrogenases assay

A colorimetric method based on the reduction of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was applied for the determination of mitochondrial dehydrogenases activity (32, 38). Briefly, mitochondrial suspension in a buffer solution (1 mM EDTA, 320 mM sucrose, and 10 mM Tris-HCl, pH=7.4), was incubated with 40 µl of MTT (0.4% w:v; 37 °C for 30 minutes, in the dark) (39, 40). Then, samples were centrifuged (10,000 g, 5 minutes) and the product of purple formazan crystals was dissolved in 1 ml dimethyl sulfoxide (DMSO). Afterwards, 0.1 ml of the dissolved formazan was added to a 96 well plate. Finally, the optical density (OD) at λ=570 nm was measured with an EPOCH plate reader (BioTek® Instruments, Highland Park, USA) (32, 38).

2.8. Reactive oxygen species (ROS) in isolated liver mitochondria

The ROS measurement was performed using the fluorescent probe DCFH-DA (25, 41-43). Briefly, isolated liver mitochondria were incubated in a respiratory buffer solution containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 20 µM Ca^{2+}, sodium succinate 5 mM, pH=7.2 (25). Following this step, DCFH-DA was added (Final concentration, 10 µM) to mitochondria and then incubated for 30 min at 37 ºC. Then, the fluorescence intensity of DCF was measured using a FLUOstar Omega® multifunctional fluorescent microplate reader ($\lambda_{excitation}$=485 nm and $\lambda_{emission}$=525 nm) (25).

2.9. Statistical analysis

Data are given as the Mean±SD. Data comparison was performed by the one-way analysis of variance (ANOVA) with Tukey's multiple comparison test as the post hoc. Differences were considered statistically significant when P-value <0.05.

3. Results

Isolated liver mitochondria were incubated with different concentrations of CA (0.01 mM-160 mM). CA treatment (0.01-10 mM) significantly enhanced mitochondrial indices of functionality in isolated mice liver mitochondria (Figure 1). It was found that CA administration (0.01-10 mM) improved mitochondrial membrane potential, promoted mitochondrial ATP metabolism, and increased mitochondrial dehydrogenases activity (Figure 1).

The mitochondrial protecting properties of CA was also assessed in a Ca^{2+}-overloaded environment. For this purpose, isolated mitochondria were incubated with Ca^{2+} (200 µM) and CA (0.01-20 mM; 30 minutes before Ca^{2+}) (Figures 2 and 3). It was found that CA supplementation (0.01, 0.05, 0.1 and 1 mM) significantly prevented Ca^{2+}-induced mitochondrial dysfunction as judged by higher ATP content, increased mitochondrial dehydrogenases activity, and prevention of mitochondrial depolarization in CA-treated groups (Figures 2 and 3). Moreover, mitochondrial permeabilization and ROS formation were significantly lower in CA-treated isolated mitochondria (Figures 2 and 3).

4. Discussion

Carnosine (CA) is a dipeptide abundantly found in different mammalian tissues. Several physio/pharmacological roles are attributed to this peptide. It has been established that CA counteracts reactive oxygen and nitrogen species (1, 10, 13, 44, 45), chelates metal ions (46, 47), and pro-

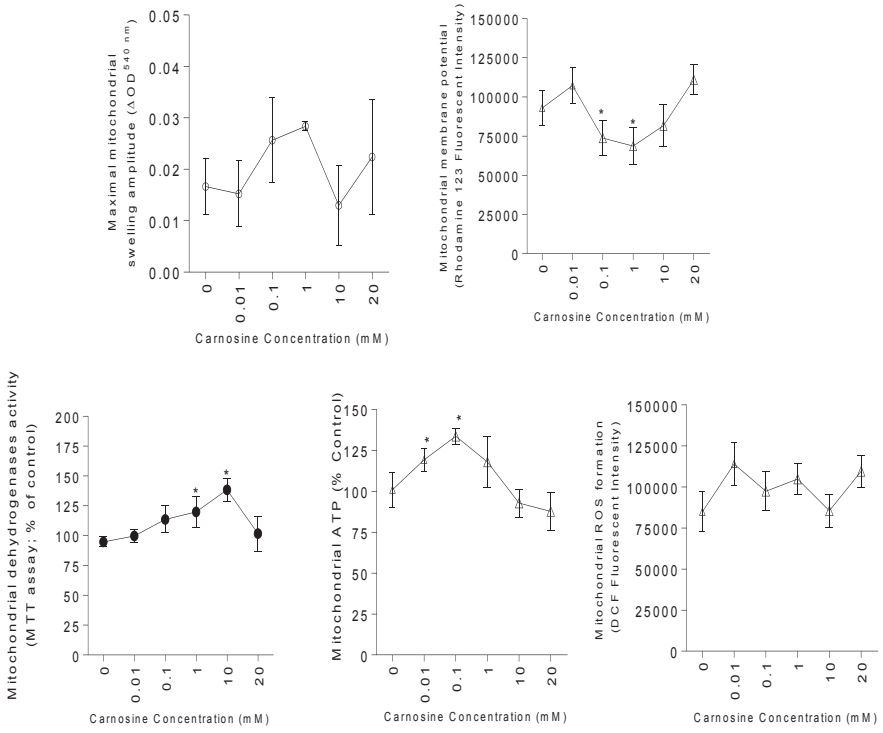

Figure 1. Concentration-response of carnosine on mitochondrial indices of functionality.
Data are given as Mean±SD (n=8).
*Indicates significantly different as compared with control (0 mM carnosine) (P<0.01).

tects biological systems against oxidative stress and its deleterious consequences (1, 10, 13, 44, 45). Another essential physiological role for CA includes intracellular pH control (48, 49). The effect of this peptide on different systems including cardiovascular, immune, and renal systems has been widely investigated (44, 50-52). Despite the tremendous physiological and pharmacological effects assigned to CA, its precise mechanism(s) of cytoprotection/toxicity remains unclear. The current study was designed to investigate the impact of various CA concentrations on cellular mitochondria as a significant target for cytoprotection/cytotoxicity.

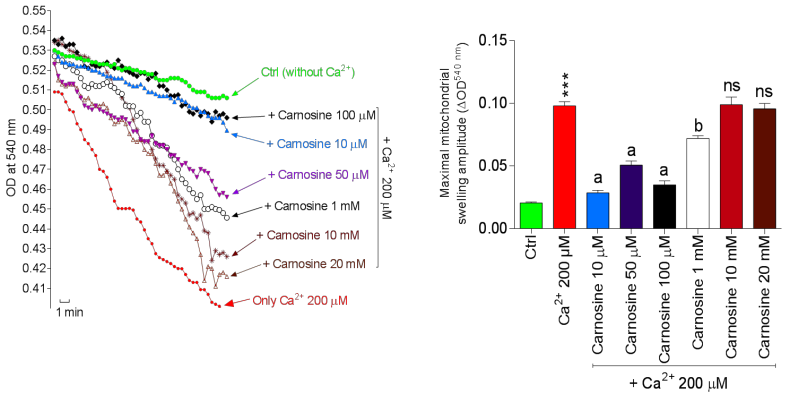

Figure 2. Calcium (Ca^{2+})-induced mitochondrial permeabilization and swelling in the presence of carnosine.
Data are given as Mean±SD (n=8).
***Indicates significantly different as compared with control (P<0.001).
[a]Indicates significantly different as compared with Ca^{2+} 200 µM (P<0.001).
[b]Indicates significantly different as compared with Ca^{2+} 200 µM (P<0.05).
ns: not significant as compared with Ca^{2+} 200 µM (P>0.05).

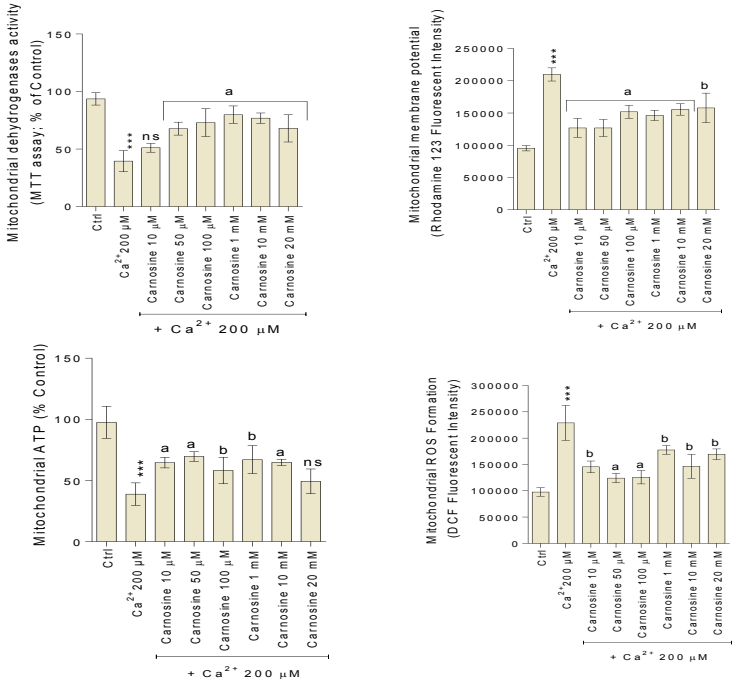

Figure 3. Effect of carnosine on Ca^{2+}-induced mitochondrial dysfunction.
Isolated liver mitochondria were incubated with Ca^{2+} (200 µM) alone or in combination with carnosine. Carnosine was added 15 minutes before Ca^{2+} challenge. Data are given as Mean±SD (n=8).
***Indicates significantly different as compared with control (P<0.001).
[a]Indicates significantly different as compared with Ca^{2+} 200 µM (P<0.001).
[b]Indicates significantly different as compared with Ca^{2+} 200 µM (P<0.05).
ns: not significant as compared with Ca^{2+} 200 µM (P>0.05).

Several investigations have mentioned that different pathological states might benefit from exogenous CA supplementation (44, 50-52). The protective effects of this peptide against nervous system disorders, aging, cardiovascular complications, and xenobiotic-induced organ injury have been reported (44, 50-52). As mentioned, CA is a well-known antioxidant and scavenger of reactive intermediates (5, 44, 53, 54). This peptide is also reported to boost antioxidant defense mechanisms and preserve cellular glutathione reservoirs (17, 50, 55). CA could efficiently protect biological targets (e.g., DNA, biomembranes lipid) against reactive species (3, 9). Although the antioxidant and reactive species scavenging properties of CA might play a significant role in its protective effects, the exact mechanism of protection provided by this peptide has not been precisely cleared in most cases. The data obtained from the current study revealed that cellular mitochondria is also a critical target for the cytoprotection provided by CA.

The involvement of CA in the regulation of mitochondria-mediated cell death has been previously mentioned in different experimental models (21-23). The data obtained from the current study revealed that carnosine (0.01 mM- 10 mM) can effectively preserve mitochondrial functionality against Ca^{2+} overload as a universal mechanism involved in mitochondria-mediated cell death (24).

The buffering capacity of CA has been mentioned as an essential feature of this peptide (56). It has been established that CA preserves cellular pH at its physiological level and prevents cell injury (56, 57). Physiologically, the buffering activity of CA becomes crucial in highly active tissues such as skeletal muscles where the acidification of the cellular environment commonly takes place (56, 57). On the other hand, it has been proposed that CA buffering capacity might play a role in enhancing mitochondrial functionality (23). Previous studies indicate that carnosine efficiently

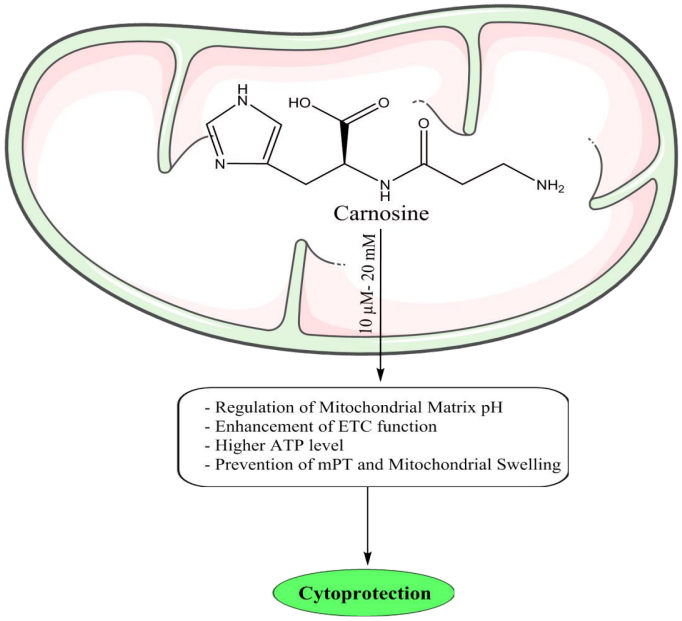

Figure 4. A schematic representation of the effect of carnosine on cellular mitochondria. The mitochondria protection or mitochondria dysfunction depends on the carnosine concentration. ETC: Electron Transfer Chain; mPT: Mitochondrial Permeability Transition.

preserves mitochondrial membrane potential (23, 58). Mitochondrial pH gradient and membrane potential are essential factors for mitochondrial function (23). Hence, chemicals which are capable of localizing in the cellular mitochondria and regulating matrix pH can conserve mitochondrial membrane potential. It has been found that CA might play a role in mitochondrial matrix pH regulation (23). In the current study, we found that mitochondrial membrane potential, as an index of H^+ gradient, was higher in CA-treated mitochondria (0.01-10 mM) (Figure 1). The buffering property of carnosine might help preserve the proper function of mitochondrial matrix localized enzymes. All these findings could indicate the importance of CA in regulating mitochondrial function and energy metabolism.

As mentioned, the relevance of mitochondrial protecting properties of carnosine against xenobiotic-induced toxicity has been proposed in different previous investigations (1, 2, 18-20). It has been mentioned that this peptide prevents oxidative stress, apoptosis, and cell death in different experimental models (19, 59-63). However, the direct effect of various concentrations of carnosine on mitochondria is obscure. In the current investigation, we found that carnosine prevented mitochondrial dysfunction against Ca^{2+} overload as the major cellular signaling molecule responsible for mitochondrial dysfunction and its associated events (24). Hence, carnosine might be applicable not only against xenobiotic-induced mitochondrial dysfunction (33, 64-70) but also against a wide range of mitochondria-linked diseases.

Previous investigations applied very high and possibly biologically-irrelevant concentrations/doses of CA (71-73). It may be possible to design more efficient similar structures which affect cellular mitochondria and energy metabolism. On the other hand, efficient mitochondria-targeting drug delivery systems might help deliver CA to the cellular power plants. Such investigations might help conquer CA bioavailability obstacles and develope carnosine and its similar structures as promising therapeutic agents against mitochondria-linked disorders.

Collectively, our data suggest cellular mitochondria as an essential target for the cytoprotective properties of CA. Indeed further research on the effects of CA on different mitochondrial components will enhance our understanding about the pleiotropic pharmacological properties of this naturally occurring peptide.

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Conflict of Interest

None declared.

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