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ORIGINAL ARTICLE

# Carnosine protects neurons against oxidative stress and modulates the time profile of MAPK cascade signaling

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**Abstract** Carnosine is a known protector of neuronal cells against oxidative injury which prevents both apoptotic and necrotic cellular death. It was shown earlier that carnosine serves as an intracellular buffer of free radicals. Using the model of ligand-dependent oxidative stress in neurons, we have shown that homocysteine (HC) initiates long-term activation of extracellular signal regulated kinase, isoforms 1 and 2 (ERK 1/2) and Jun N-terminal kinase (JNK) which corresponds to exitotoxic effect resulting in cellular death. L-Carnosine ($\beta$-alanyl-L-histidine) protects neurons from both excitotoxic effect of homocysteine and cellular death. Its analogs, $\beta$-alanyl-D-histidine (D-carnosine) and L-histidyl-$\beta$-alanine, restricted accumulation of free radicals and delayed activation of ERK1/2 and JNK in neuronal cells, but did not promote neuronal viability.

**Keywords** Hyperhomocysteinemia · Carnosine · Excitotoxicity · MAPK · Neuroprotection

## Introduction

Carnosine is a known antioxidant that can limit accumulation of reactive oxygen species, which allows protecting neurons from oxidative damage, thus preventing both necrosis and apoptosis (Boldyrev 2011). Since the latter kind of cell death does not depend directly on free radicals and is regulated by MAP kinase cascade signaling, it is interesting to study the ability of carnosine to affect the state of cell signaling cascade under oxidative stress.

It is known that the time profile of activation of intracellular kinases is crucial for cell survival/adaptation. Short-term activation of extracellular signal regulated kinase, isoforms 1 and 2 (ERK 1/2) accompanying with inhibition of Jun N-terminal kinase (JNK) enhances cell survival (Navon et al. 2011) whereas long-term activation of these kinases triggers apoptosis (Glotin et al. 2006). The basis of such effect is an ability of this signaling cascade to regulate expression of early response genes, so that the cell genome is a sensor for red/ox state of the cell, which is affected by intracellular free radicals often determined as reactive oxygen species (Oktyabrsky and Smirnova 2007).

Some evidence in the literature suggests that carnosine is able to affect expression of individual enzymes, to inhibit accumulation of pro-inflammatory factors and to increase endogenous antioxidant defense system (Son et al. 2008; Calabrese et al. 2008; Boldyrev 2009). All this was a prerequisite for evaluating the effect of carnosine on the state of ERK 1/2 and JNK in cerebellum granule neurons under oxidative stress caused by their incubation with homocysteine (HC).

Homocysteine is the non-protein sulfur-containing amino acid which is an intermediate in the metabolism of methionine; its normal level in the blood of healthy donors is 10–15 μmol/L. Hyperhomocysteinemia is characterized by increased levels of HC in the blood to 100–500 μmol/L, which is a risk factor for cardiovascular and neurodegenerative diseases, initiating recurrent heart attack and stroke (Sacco et al. 1998), accompanies and exacerbates senile

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dementia, Parkinson's and Alzheimer's diseases (Seshadri et al. 2002). Hyperhomocysteinemia causes a decrease in the volume of the hippocampus and cerebral cortex, even in elderly people not suffering from neurodegenerative diseases (den Heijer et al. 2003). The stroke which accompanied with hyperhomocysteinemia results in significant increase in brain infarct area and stimulation of neuronal death in the damaged region of brain (Poddar and Paul 2009).

In the HC toxicity the ionotropic glutamate receptors of *N*-methyl-D-aspartate (NMDA) class, NMDARs are involved (Boldyrev 2009). Interaction of HC with NMDARs activates several signaling cascades within the cell, including the MAP signaling cascade involved in apoptosis regulation. Thus, oxidative stress caused in neuronal cells by hyperhomocysteinemia reflects significant mechanisms of brain damage during development of neurodegenerative diseases.

Previously, we demonstrated that carnosine effectively protects animals from HC toxicity in vivo (Makhro et al. 2008; Boldyrev 2009), but mechanism of its action remained unclear. In this study, we investigated the effect of carnosine on MAP kinase cascade of cerebellum neurons under HC-induced oxidative stress in vitro.

## Materials and methods

All experiments were conducted on 10-day-old laboratory Wistar rats in accordance with the international rules for work with laboratory animals (http://www.nap.edu/books/0309083893/html/R1.html). Freshly isolated suspension of cerebellum granule cells was used in flow cytometric measurements of cell viability, and primary culture of cerebellum neurons was used to determine activation of intracellular kinases, in accordance with technical constraints of above methods.

Suspension of cerebellar granule cells was isolated from cerebellum of 10-day-old rats (Boldyrev et al. 1999). The animals were decapitated, cerebellum was removed, cut to thin slices in cold Tyrode's solution (NaCl 130 mM, KCl 4 mM, $CaCl_2$ 1,8 mM, $NaHCO_3$ 11 mM, $MgCl_2$ 1 mM, $NaH_2PO_4$ 0,4 mM, glucose 5,5 mM) (PanEko, Russia), and then treated with collagenase (Wako collagenase, 2 mg/mL, 400 U) prepared on Tyrode's solution. After 30 min incubation with collagenase at 32°C, cerebellum pieces were washed twice, triturated using Pasteur pipette, and filtered through 53 μm teflon filter. Prior to experiments, the cells were restituted for 30 min at 37°C. Freshly prepared suspension of cerebellum granule cells was contaminated with the cells of other nature which were removed from analysis using the gate selecting population of neurons (Boldyrev et al. 1999).

Primary culture of cerebellar granule cells was prepared as follows. Cerebellums of 10-day-old rats were washed with cold Hank's solution (PanEko), and incubated for 20 min with 0.05% trypsin solution (PanEko), then the cells were washed and cultured for 11 days on Neurobasal™-A medium with 2% Supplement B-27 as a substitute for serum (Invitrogen, USA), GlutaMax™ (Invitrogen), 50 U/mL penicillin, 50 U/mL streptomycin and 20 mM KCl (5% $CO_2$ at 37°C). One hour before the experiment, the culture medium was replaced for so-called "starving medium" (Hank's solution). The resulting culture contained about 95% living neuronal cells. To activate signaling pathways, cells were incubated for 2.5–40 min with 500 μM of HC in the presence of co-activator of NMDARs, glycine (in the presence or absence of the dipeptides tested). The HC concentration used in these experiments was found in previous experiments as sufficient for realization of exitotoxic action (Makhro et al. 2008). After subsequent incubation, the cells were analyzed by western blotting.

### Determining the free radicals and the number of necrotic cells

To determine the level of free radicals, freshly prepared suspension of cerebellar granule cells was incubated with fluorescent probe DCF-DA (2,7-dihlorodihydrofluoresceine diacetate, Molecular Probes, USA) in a final concentration of 100 μM for 40 min at 37°C in the dark. The cells were incubated with homocysteine (in the presence or absence of the dipeptides), and analyzed using BD FACS Calibur flow cytometer (Becton–Dickinson, USA), gating the population corresponding to neuronal cells by size (about 10 μM) and expression of NMDARs (Boldyrev et al. 1999; Makhro et al. 2008). The mean fluorescence of DCF was used as an indicator of free radical levels within the cells. The percentage of necrotic cells in the gated cell population was measured after staining the samples with 10 μM propidium iodide (PI, Sigma, Germany) added 1 min before measurements. Data were analyzed via WinMDI software. Mean ± SEM ($n = 3$) was used for statistical comparison using ANOVA. Differences were considered significant when $p < 0.05$.

### Measurement of protein kinases (Poddar and Paul 2009)

Activity of intracellular kinases that regulate cell viability was measured in primary culture of cerebellum neurons as the ratio of phosphorylated forms of subsequent kinase to its endogenous control (the overall level of actin in the cells). After incubation, cells with the ligand were washed with cold Hank's solution, and lysed in RIPA-buffer (Tris 50 mM, NaCl 150 mM, SDS 0.1%, sodium deoxycholate 0.5%, Triton X100 1%, cocktail of protease inhibitors)

(Sigma-Aldrich, USA). Lysates were subjected to electrophoresis in polyacrylamide gel with 10% separating and 6% concentrating gels. The Prestained Protein Molecular Weight Marker (Fermentas, Germany) was used to calibrate molecular mass of the spots. After electrophoresis, proteins were subjected to electrotransfer to PVDF membranes (ThermoScientific, USA). Detection of proteins was carried out by the primary antibodies anti-phospho-ERK 1/2 (Thr202/Tyr204) (1:1,000, Cell Signaling, USA), or antibodies specific for total actin (1:2,000, Cell Signaling, USA) or anti-phospho-JNK (Thr183/Tyr185) (1:2,000, Cell Signaling, USA) and secondary antibodies conjugated with peroxidase (1:1,000, Cell Signaling, USA). Then the membranes were stained by enhanced chemiluminescence (ECL). The data were analyzed using the program Bio-Rad Quaintity One™. Mean ± SEM ($n = 3$) were used for statistical comparison using ANOVA. Differences were considered significant when $p < 0.05$.

### Carnosine and its analogs

In the experiments, L- and D-carnosine as well as their structural analogs, L-histidyl-$\beta$-alanine, were used. L-Carnosine was purchased from "Hamari Chem., Ltd" (99% purity), D-carnosine (97.5% purity) and L-histidyl-$\beta$-alanine (98% purity) were synthesized by routine peptide synthesis. Purity of the substances was confirmed by HPLC. Each of the compounds was used from the 10 mM stock solution with pH adjusted to 7.4 by 0.1 N HCl. In order to load the cells with the dipeptides, 30 min pre-incubation of cell suspension with 1 mM of L-carnosine, D-carnosine or L-histidyl-$\beta$-alanine (final concentration) was used before adding of HC to cell samples.

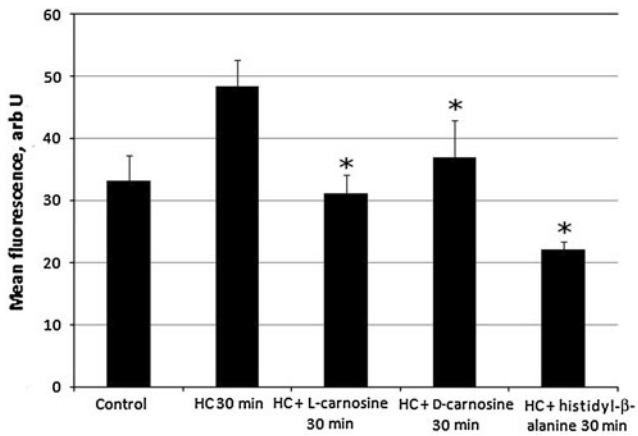
## Results

In our experiments, 30 min pre-incubation of cerebellum granule cells with 500 μM HC caused a significant increase in the intracellular levels of free radicals and of necrotic cell death (Fig. 1). However, pre-incubation of cells with L-carnosine completely prevents the development of oxidative stress in neurons, and protects neurons from necrotic death, despite the presence of HC. D-carnosine and L-histidyl-$\beta$-alanine used in similar conditions, although reduced the accumulation of free radicals in the treated cells, but did not prevent neuronal death (Fig. 1).

It is known that HC causes oxidative stress in neuronal cells because of its activating effect on both ionotropic and metabotropic glutamate receptors (Gu et al. 2010), thus creating conditions for exitotoxic cell death (Boldyrev et al. 2011). In our experiments, toxic effect of HC was realized only through NMDARs, since incubation of the cells with HC in the presence of 10 μM MK-801, inhibitor of these receptors completely prevented its effect (Boldyrev et al. 2011).

Incubation of cerebellum granule cells with HC results in the activation of intracellular signaling cascades, including MAP signaling cascade involved in the regulation of cell survival/death (Boldyrev 2003; McCurbey et al. 2006; Wang et al. 2007). We analyzed time-dependent profiles of MAPK family enzymes, ERK 1/2 and JNK in cerebellum neurons incubated with HC.

The data presented in Fig. 2 shows that HC causes fast and long lasting activation of ERK 1/2. Long-term activation of this MAPK is known to contribute to cell death (Glotin et al. 2006; Ries et al. 2008). Pre-incubation of the cells with dipeptides tested significantly alters the time profile of activation of ERK 1/2 induced by HC. In the



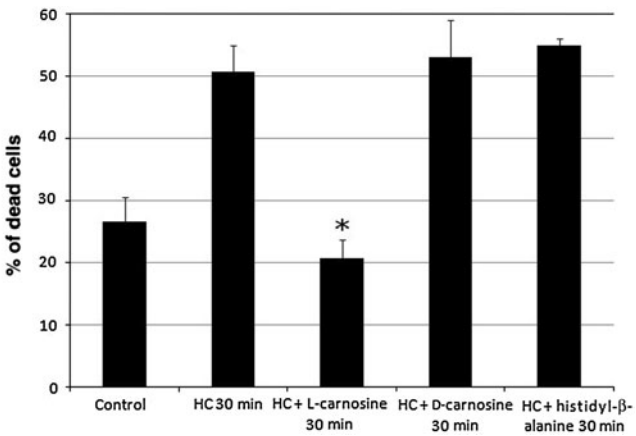




**Fig. 1** Incubation of rat cerebellar granule cells with HC (500 μM for 30 min) induces accumulation of free radicals (*left*), and increases the number of necrotic cells, labeling with propidium iodide (*right*). The presence of L-carnosine, D-carnosine or L-histidyl-$\beta$-alanine (1 mM each) prevents the accumulation of free radicals, but only the presence of L-carnosine protects neurons from necrotic death. *Asterisk* marks significant difference compared to sample containing HC ($p < 0.05$)

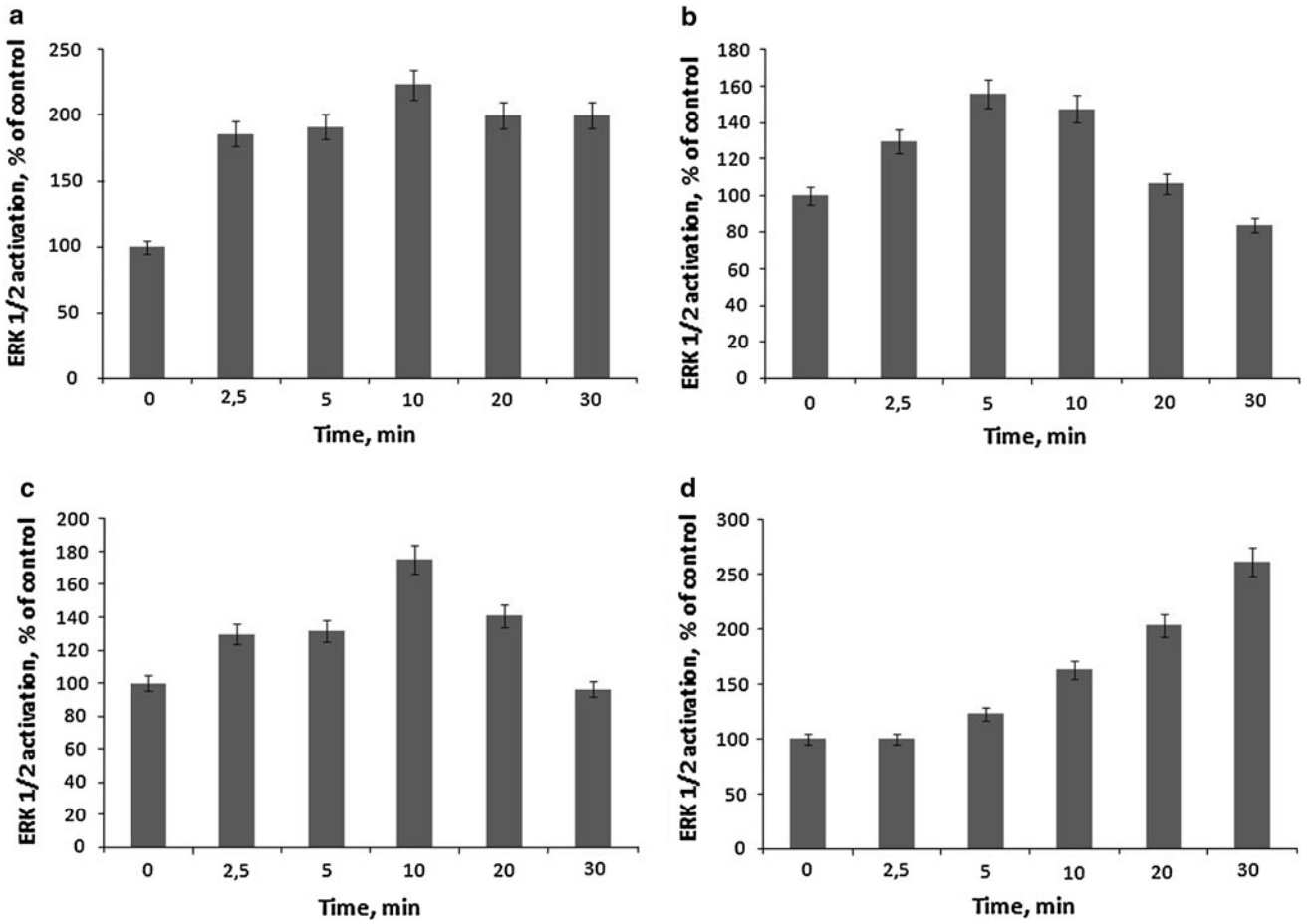



**Fig. 2** Time profile of ERK 1/2 activation induced by HC (500 μM) to intact cells (**a**) or to cells pre-loaded with L-carnosine (**b**), D-carnosine (**c**) or L-histidyl-$\beta$-alanine (**d**) (1 mM each)

presence of L-and D-carnosine activation of ERK 1/2 is slower, does not reach the same level as in the presence of HC alone, and has a short-term time profile. In the presence of L-histidyl-$\beta$-alanine, activation of ERK 1/2 is evidently delayed in time.

Incubation of cerebellum neurons with HC also causes an activation of other kinase of the same family, JNK. At the same time, incubation with HC of the neurons pre-loaded with any of the dipeptides tested causes no JNK activation (Fig. 3).

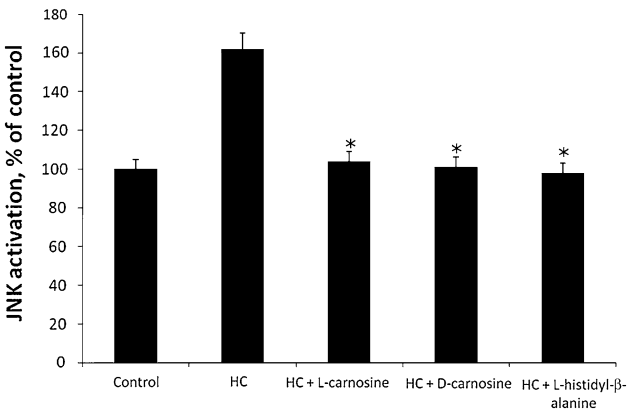



**Fig. 3** Effect of HC (500 μM for 10 min) on JNK activity in rat cerebellar granule cells in the absence or presence of the dipeptides (as noted). *Asterisk* marks significant difference compared to sample containing HC ($p < 0.05$)

## Discussion

We have already demonstrated a protective ability of carnosine during hyperhomocysteinemia in vivo (Boldyrev 2009). The results presented here reveal possible mechanisms of its action. Protective effect of carnosine can be realized via at least two directions. First, it is able to prevent accumulation of free radicals caused by the activation of NMDARs (Boldyrev et al. 1999; Makhro et al. 2008). This effect reflects the well-known property of carnosine to act as a direct antioxidant (Boldyrev et al. 1987; Aruoma

et al. 1989; Boldyrev 1993). Secondly, carnosine can modulate intracellular signaling cascades.

It is known that HC activates in neurons the same class of ionotropic glutamate receptors as NMDA does (Boldyrev 2009). This explains why addition to the samples of NMDARs blocker, MK-801 completely prevents effect of HC on DCF fluorescence and activation of ERK 1/2 (Boldyrev et al. 2011). However, activation of NMDARs by two different ligands, NMDA and HC, is different. NMDA, as well as glutamate activates the receptor in a short-term manner which corresponds to reversible interaction of the ligand with receptor protein. The nature of interaction of receptor with HC has not been yet investigated, but it is known that HC is able to form thiolactone which can easily attack ε-amino groups of lysine of diverse protein molecules (Jakubowski et al. 2000; Perla-Kaján and Jakubowski 2010). As a result of this attack, *N*-homocysteinylated proteins are formed with impaired function. If *N*-homocysteinylation also takes place in our case, it may cause the long-term activation of protein kinase cascade.

An interesting question is that the presence of carnosine results in the transformation of time profile of ERK 1/2 activation from the long-term into the short-term one, which is typical for activation of receptors by NMDA (Boldyrev et al. 2011). This feature, along with the prevention of activation of JNK (Fig. 3) corresponds to formation of anti-apoptotic signal (Navon et al. 2011). The observed ability of carnosine to modify the HC effect may be linked to the chemical feature of carnosine that can repair proteins modified via lysine ε-amino groups as it was found earlier for modification of actin by toxic aldehydes (Kuleva and Kovalenko 1997).

Similar ability to modify the activation of ERK 1/2 kinase in our experiments has been demonstrated by both D-carnosine and L-histidyl-β-alanine. In addition, they prevented activation of JNK caused by homocysteine. It is possible that such effect is closely related to their restrictive action on accumulation of free radicals by cerebellum granule cells. At the same time, effective prevention of necrotic neuronal death was characteristic of only L-carnosine. This argues for the importance of specific amino acid arrangement in these dipeptides for the exerted biological activity.

As it is seen from Fig. 1 (left), all three dipeptides decrease the stationary levels of free radicals within the cerebellum neurons, thus suggesting that all they can penetrate cell membrane, and be accumulated inside the cells. In agreement with this, all the dipeptides tested modify the time profile of ERK1/2 (while by a different manner, see Fig. 2), and suppress JNK activity (Fig. 3). Nevertheless, only L-carnosine promotes the survival of neuronal cells. In other words, our data suggest two different directions of action of these regulatory dipeptides on neuronal cells, one is related to protection against necrotic death (Boldyrev et al. 1999; Boldyrev 2007), and another to modulation of intracellular signaling mechanisms (Boldyrev et al. 2011). It is important that only natural dipeptide L-carnosine demonstrates an ability to modulate both sides of cellular life.

The data presented allow us to consider carnosine as a potential drug that can reduce damage to the nervous system in patients with hyperhomocysteinemia.

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