Pergamon

PII: S0306-4522(99)00273-0

Neuroscience Vol. 94, No. 2, pp. 571–577, 1999
Copyright © 1999 IBRO. Published by Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0306-4522/99 $20.00+0.00

CARNOSINE PROTECTS AGAINST EXCITOTOXIC CELL DEATH INDEPENDENTLY OF EFFECTS ON REACTIVE OXYGEN SPECIES

A. BOLDYREV,*†‡ R. SONG,†‡ D. LAWRENCE†‡ and D. O. CARPENTER†‡§

*International Center for Biotechnology and Center for Molecular Medicine, MV Lomonosov Moscow State University (Department of Biochemistry, School of Biology), 119899 Moscow, Russia

†Department of Environmental Health and Toxicology, School of Public Health, University at Albany, Rensselaer, NY 12144-3456, U.S.A.

‡Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201-0509, U.S.A.

Abstract—The role of carnosine, N-acetylcarnosine and homocarnosine as scavengers of reactive oxygen species and protectors against neuronal cell death secondary to excitotoxic concentrations of kainate and *N*-methyl-D-aspartate was studied using acutely dissociated cerebellar granule cell neurons and flow cytometry. We find that carnosine, N-acetylcarnosine and homocarnosine at physiological concentrations are all potent in suppressing fluorescence of 2′,7′-dichlorofluorescein, which reacts with intracellularly generated reactive oxygen species. However, only carnosine in the same concentration range was effective in preventing apoptotic neuronal cell death, studied using a combination of the DNA binding dye, propidium iodide, and a fluorescent derivative of the phosphatidylserine-binding dye, Annexin-V.

Our results indicate that carnosine and related compounds are effective scavengers of reactive oxygen species generated by activation of ionotropic glutamate receptors, but that this action does not prevent excitotoxic cell death. Some other process which is sensitive to carnosine but not the related compounds is a critical factor in cell death. These observations indicate that at least in this system reactive oxygen species generation is not a major contributor to excitotoxic neuronal cell death. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: cerebellar granule cells, cell death, flow cytometry, NMDA, kainate, apoptosis.

Excitotoxic concentrations of glutamate or its analogs, *N*-methyl-D-aspartate (NMDA) and kainate, cause neuronal cell death.[35,46] Neuronal death can be either necrotic (cell swelling with accumulation of NaCl) or apoptotic (programmed cell death with cell shrinkage), and both forms of cell death have been described in excitotoxicity.[39,46] Some have suggested that apoptotic transformation of neurons may be the basis of several of the neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis.[11,28]

While apoptosis can be triggered by a variety of factors, one of particular importance is the generation of reactive oxygen species (ROS).[21,38,50] ROS are generated following oxidative stress of the cell,[43] through activation of excitatory amino acid receptors[37] and by a variety of other mechanisms.[1] There is evidence that superoxide anion or hydroxyl radical may be the immediate cause for apoptosis of neurons.[34] Consistent with this conclusion is evidence that scavengers of hydroxyl radicals protect against apoptotic transformation in cultured cells.[30] However, it remains unclear whether the hydroxyl radicals are indeed the cause of apoptosis. Others have cautioned that glutamate-induced apoptosis in cultured cerebellar granule cells is not prevented by inhibition of xanthine oxidase, and that vitamin E and its analogs are only partially effective.[10]

There are endogenous antioxidant defense systems, which include a number of enzymes, such as superoxide dismutase, catalase and glutathione peroxidases, as well as several non-enzymatic antioxidants (for example, carotenes, tocopherols, ascorbic acid, and taurine).[1] We have focused this investigation on the role of carnosine and related compounds as antioxidants and protective agents against excitotoxic cell death.

Carnosine (β-alanyl-L-histidine) is present in excitable tissues at relatively high concentrations, but to date its function(s) are unclear. Carnosine is present in mammalian brain at a concentration between 0.7–2.0 mM, depending on the brain region.[26] Homocarnosine is a closely related substance, and both carnosine and homocarnosine exist as such and as N-acetylated derivatives.[47]

There has been considerable debate for many years as to the physiologic function(s) of carnosine. Carnosine has been considered as a mobile organic pH-buffer.[3] Because carnosine binds to metals such as copper and zinc, it has also been considered to have a role as a metal chelator.[20,49] Since it is asymmetrically distributed in brain, and is particularly elevated in the olfactory bulb,[27] it has been proposed to be a neurotransmitter.[32] However, this hypothesis has not been supported because of the lack of carnosine receptors in the brain[4] and the lack of electrophysiological responses to applied carnosine.[8,25] Finally, carnosine and related compounds have also been shown to be powerful hydrophilic ROS scavengers.[5,20] Carnosine and glutamate appear to be co-localized in presynaptic terminals in brain,[4,41] which is consistent with the hypothesis that carnosine has a role as an endogenous agent to protect against glutamate excitotoxicity secondary to ROS generation.

We have investigated the actions of carnosine, homocarnosine and N-acetylcarnosine on acutely isolated cerebellar granule cells, studied in a flow cytometer where we can monitor cell death upon application of the excitatory amino acids, and measure ROS with fluorescent dyes.

§To whom correspondence should be addressed. Tel.: + 1-518-5252660; fax: + 1-518-5252665.
E-mail address: carpent@cnsvax.albany.edu (D. O. Carpenter)

Abbreviations: AGE, advanced glycation end product; DCF-DA, 2′,7′-dichlorofluorescein diacetate; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; NMDA, *N*-methyl-D-aspartate; PI, propidium iodide; ROS, reactive oxygen species.

EXPERIMENTAL PROCEDURES

Animals

Wistar rats (Wadsworth Center breeding colony; 10–12-days-old) weighing 18–22 g were used in all experiments. All animal experiments were carried out in accordance with the National Institute of Health guidelines for the care and use of laboratory animals and all efforts were made to reduce suffering.

Cell preparation and treatment

Cerebellar granule cell neurons were obtained as previously described.[33] Briefly, the cerebellum was removed, cut into slices, incubated for 1 h in dispase and the neurons dissociated by gentle trituration. Debris was removed by filtration through a 53-μm mesh. The neurons were resuspended in Tyrode's solution (NaCl, 145 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; glucose, 5 mM; HEPES, 5 mM, pH 7.3) and incubated for 1 h prior to study at 33–34°C.

Three fluorescent dyes were used: the ROS sensitive 2′,7′-dichlorofluorescein diacetate (DCF-DA); a membrane impermeant DNA binding dye, propidium iodide (PI), which is used to detect cells that have lost membrane integrity; and fluorescein isothiocyanate (FITC)-labelled Annexin-V, which was used to detect cells early in the process of apoptosis.

Changes in ROS concentration were measured by use of DCF-DA.[33] This dye is accumulated in living cells when the acetate groups are cleaved, thus transforming DCF-DA to DCF-H_2. In the presence of ROS, DCF-H_2 undergoes oxidation to DCF, which is fluorescent. Dead cells were detected by use of PI, a DNA-binding dye which is excluded from cells with intact plasma membranes. Thus, the fluorescence of PI increases when cells lose membrane integrity.[12] Both PI and DCF are excited at 488 nm and show fluorescence maxima at 510–520 nm (DCF) and 575–580 nm (PI). Since the emission spectra do not overlap significantly it was possible to monitor the fluorescence of both dyes simultaneously. Cells were incubated with DCF-DA for 60 min at a final concentration of 100 μM.[33] Then the excitatory amino acid agonists (NMDA or kainate) were added at various concentrations for various periods of time. PI (final concentration 10 μg/ml) was added to the samples 1–2 min before measurements were taken. Under these circumstances there was an unchanged fluorescence signal for both DCF and PI for at least 120 min in the absence of NMDA or kainate.

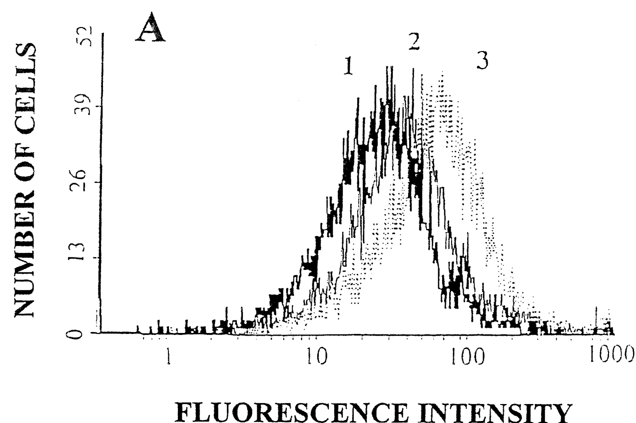
In order to distinguish necrotic from apoptotic cell death we used FITC-labeled Annexin-V (Apoptotic Detection Kit no. KNX-020, R and D System). This system allows us to detect cells in early apoptosis because the Annexin-V specifically binds to phosphatidylserine. One of the earliest events in apoptosis is the movement of phosphatidylserine from the inner to the outer membrane leaflet before the membrane loses integrity.[48] By plotting PI fluorescence against Annexin-V fluorescence one can follow the progression to cell death by each of the two mechanisms (necrosis or apoptosis).

Flow cytometry studies

All flow cytometric measurements were made using an EPICS ELITE ESP flow cytometer. Cells were suspended in 2 ml containing $1.5–2 \times 10^6$ cells. Unless otherwise specified, carnosine, homocarnosine or N-acetylcarnosine were added to the cells at the beginning of the 1 h incubation period at a concentration of 10 mM. For controls the same concentration of HEPES buffer was added. After incubation for recovery from dissociation and incubation with DCF-DA, cells were diluted 10-fold with normal Tyrode's solution before kainate or NMDA were added. For comparison some studies were done with carnosine added immediately before study, without preincubation.

Reagents

Carnosine was obtained from St Petersburg Drug Medical Factory (Russia), and had a purity of 99%. Homocarnosine was purchased from Sigma. N-acetylcarnosine was synthesized in the Moscow Laboratory by V. Kh. Shavratsky, and found to be 99.7% pure by high-performance liquid chromatography. All fluorescent probes were purchased from Molecular Probes. All other chemicals were purchased from Sigma.



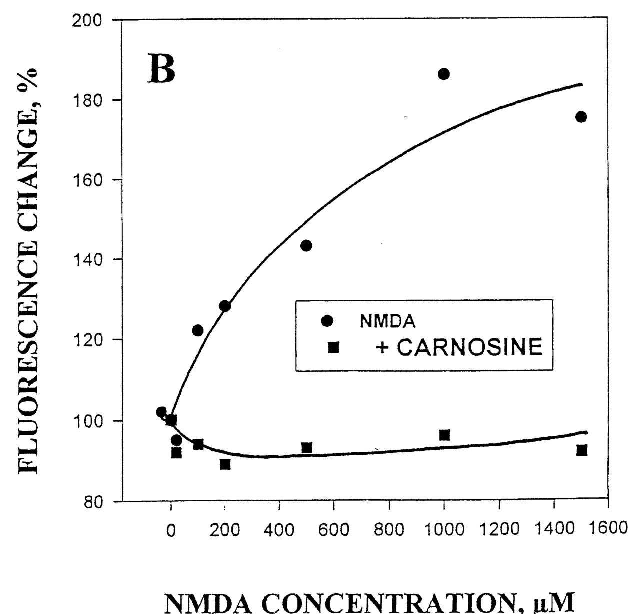


Fig. 1. Effect of carnosine on DCF fluorescence of cerebellum granule cells stimulated by NMDA. (A) Log mean DCF fluorescence of control cells (1), after incubation with 250 μM NMDA for 10 min (3), and after 250 μM NMDA for 10 min following 1 h preincubation with carnosine (2). (B) Plot of DCF fluorescence from a different but similar experiment as a function of NMDA concentration.

Statistical analysis

All parameters measured are expressed as mean ± S.D. calculated from no less than three independent measurements. Statistical analysis was carried out using routine computer programs, and *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

Exposure of DCF-loaded cerebellar granule cells to various concentrations of NMDA or kainate resulted in an increase in DCF fluorescence, which indicates an increase in the intracellular concentration of ROS. When applied at equal concentrations, NMDA was somewhat more potent than kainate. However, when neurons were pre-loaded with carnosine the mean fluorescence increase was markedly reduced. This is shown for NMDA in Fig. 1, where part A shows log raw data traces of the DCF fluorescence in control (curve 1), in the presence of NMDA (250 μM) (curve 3) and in the

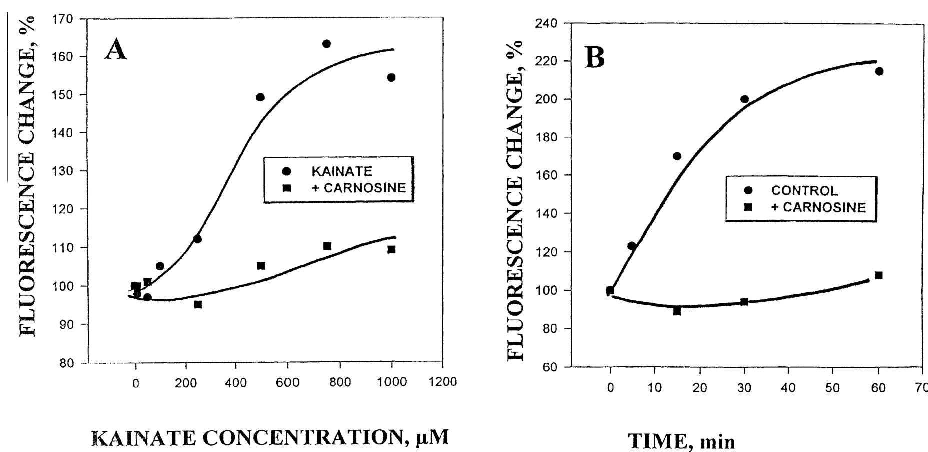


Fig. 2. Effect of carnosine on kainate stimulated fluorescence of DCF-loaded neurons. (A) Concentration dependence (10 min exposure at each concentration of kainate). (B) Time dependence in the presence of 2 mM kainate.

presence of NMDA (250 μM) but after preincubation in 10 mM carnosine for 1 hr (curve 2). Figure 1B shows the dose dependence for various concentrations of NMDA. In the absence of carnosine the DCF fluorescence intensity increased with NMDA concentration over the range 100–1000 μM. However, when the cells were preincubated with carnosine for 1 h prior to addition of NMDA the increase in DCF fluorescence was prevented. The baseline fluorescence was even slightly reduced in the presence of carnosine in the experiment illustrated in Fig. 1B.

Figure 2 shows a similar dose relationship of DCF fluorescence with kainate and the suppression of this fluorescence by carnosine (Fig. 2A), and also demonstrates the time-dependent increase in fluorescence in response to 2 mM kainate (Fig. 2B). Both NMDA and kainic acid effects were characterized by clear time and dose dependencies. For both agonists, the maximal levels of fluorescence were achieved at a concentration of 1 mM after a 30-min exposure. Carnosine reduced the mean fluorescence without alteration of the time-course.

While we did not directly determine the intracellular carnosine concentration after 1 h preincubation, we conclude that carnosine has accumulated within the cells and acts to quench intracellular ROS generated by the externally applied excitatory ligands. This conclusion is consistent with the observations that washing the cells twice after preincubation with carnosine did not alter the effectiveness of carnosine as a ROS scavenger, while addition of carnosine without preincubation together with NMDA or kainate was much less effective in reducing ROS fluorescence. Furthermore, after preincubation for 1 h in carnosine the neurons showed a significantly lower level of DCF-induced fluorescence than even in the absence of NMDA or kainate. A carnosine transporting protein has recently been demonstrated in cerebellar neurons,[51] and may mediate this uptake.

The carnosine derivatives, homocarnosine (γ-amino-butyryl-histidine) and N-acetylcarnosine, also possess antioxidant properties.[2,14] Table 1 shows the actions of these substances in reducing DCF fluorescence generated by incubation of cerebellar granule cells with 2 mM kainate. Homocarnosine and N-acetylcarnosine were equally as effective as carnosine in suppressing the DCF fluorescence increase after exposure to either NMDA or kainate, although these compounds did not decrease the initial level of DCF fluorescence.

Table 1. Effect of carnosine and related compounds on kainate stimulated fluorescence of dichlorofluorescein-loaded neurons

Conditions	Fluorescence (arbitrary units)	% Inhibition
Control neurons	58 ± 4	
With kainate (2 mM for 1.5 h)	193 ± 12*	0
Neurons with kainate (2 mM for 1.5 h) after preincubation with		
Carnosine	123 ± 14†	37
Homocarnosine	127 ± 20†	35
N-Acetylcarnosine	123 ± 11†	37

*Indicates significantly different from control with $P < 0.01$.
†Indicates significantly different from both control and kainate with $P < 0.01$.

Figure 3 shows flow cytometric analysis of PI labeling in neurons in control (A), in presence of NMDA (200 μM for 30 min) (B) and similar NMDA with carnosine. In logarithmic plots of fluorescence intensity against cell number, three sub-populations of cerebellar granule cells could usually be distinguished. The large sharp peak on the right (peak III) represents neurons heavily stained with PI, indicating that they have lost membrane integrity and have accumulated significant amounts of dye. The broader peak on the left (peak I) represents living neurons which exclude most of the dye. Finally, there is a broad intermediate peak (peak II) which has an order of magnitude less PI labeling than seen with the dead cells, but still have more labeling that the cells in the major peak of healthy cells. This intermediate population of cells has been characterized as being living but damaged[9] on the basis of the observation that the intermediate peak appears and/or grows following exposure to neurotoxic agents, and then decreases as peak III increases. This sub-population consisted of up to 16% of the cells in control in the example shown in Fig. 4, but increased to 22.9% in the presence of a relatively low concentration of NMDA. This is at a point in time when the percentage of cells in peak III has not yet increased. It appears that NMDA (and kainate) stimulates the transition of sub-population peak I into peak II and after a longer period to peak III. Carnosine appears to have a selective effect on this intermediate population. As shown in Fig. 3C, peak II is not apparent in the presence of carnosine. The observation that peak II is absent in the presence of carnosine is consistent with the possibility that carnosine

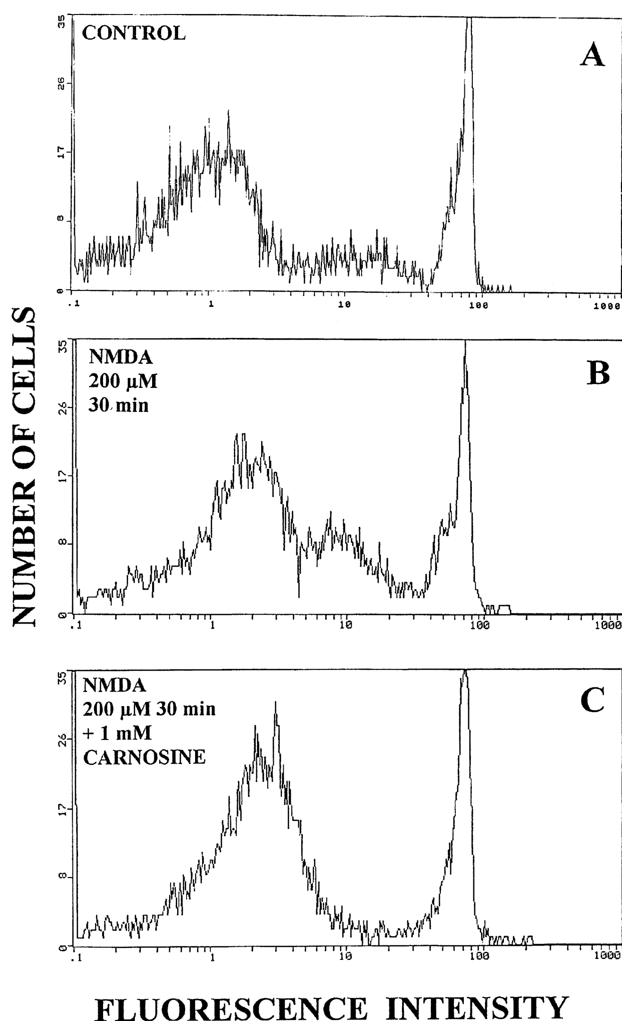


Fig. 3. Distribution of PI fluorescence intensity in neuronal cells before (A) and after 30 min exposure to 200 μM NMDA in the absence (B) and presence (C) of carnosine. Three peaks are seen in A and B, noted as I, II, and III. Peak I contained 63.9% of cells in A, 54.5% in B and 78.2% in C, while peak II was 16% (A) and 22.9% (B). Peak III was 18.5% (A), 22.9% (B) and 21.8% (C). Peak I represents healthy cells, peak II damaged cells and peak III dead cells that stain heavily for PI.

prevents cellular damage by scavenging ROS which ultimately kill cells and move them into peak III, where membrane integrity is lost. Carnosine appears to promote recovery of damaged cells in that what was in peak II is incorporated into peak I.

Table 2 shows pooled experimental results from experiments like those illustrated in Fig. 3, and confirms the pattern that carnosine reduces peak II and increases peak I to levels even beyond that seen in the control. With regard to ROS levels, the cells in peak II showed somewhat greater DCF fluorescence that those cells in peak I. There was little or no fluorescence in peak III, consistent with leakage of the DCF from the cell when membrane integrity is lost.

Since ROS generation has been implicated as a causative factor in neuronal cell death, whether by necrosis or apoptosis,[15,46] we initiated experiments shown in Fig. 4, designed to determine the mode of cell death after NMDA and kainate. This is possible because of the development of fluorescent-labeled Annexin-V. Annexin-V binds to phosphatidylserine, which is normally a constituent of only the inner membrane leaflet.[36] One of the first events in apoptosis is the movement of phosphatidylserine from the inner to the outer membrane leaflet. Therefore, one can identify those cells beginning the process of apoptosis by measuring extracellular Annexin-V labeling at a time when membrane integrity has not yet been compromised and the cells do not stain with PI. If one plots the PI fluorescence (*Y*-axis) against the Annexin-V fluorescence (*X*-axis) one can distinguish four quadrants, reflecting neurons in four different states. The cells in the lower left quadrant (P3) do not label with either probe, and are healthy. The cells in the upper left (P1) label with PI but not Annexin-V, and are cells that have lost membrane integrity via necrosis. In contrast, those cells in the lower right quadrant (P4) label with Annexin-V but not PI. These cells are early in the process of apoptosis. Finally, those cells in the upper right quadrant (P2) are labelled with both probes. One cannot definitively identify the means of death of cells in this quadrant, since it is possible that the Annexin-V labeling was through entry into the cell and binding to phosphatidylserine in the inner leaflet.

Figure 4 shows such a display of PI versus Annexin-V fluorescence. In the control most neurons are healthy and in P3, although 16.3% of cells are P1 and small numbers in P2 and P4. After a prolonged incubation with kainate (B) there is a clear increase in the percentage of neurons labeling with Annexin-V. Those neurons in P4 are now 14.8% of the total cells, which indicates that kainate promotes cell death by apoptosis. Preincubation with carnosine reduces the number of cells labeled with Annexin-V.

Table 3 shows results of pooled experiments in which carnosine, homocarnosine and N-acetylcarnosine were tested for efficacy in preventing apoptotic and/or necrotic cell death upon exposure to kainate. Carnosine was effective in preventing loss of cells from P3, and showed a highly significant and specific effect in reducing those neurons labeled with Annexin-V, whether or not they labeled with PI. However, neither N-acetylcarnosine and homocarnosine had any significant effect on the distribution of cells among the four quadrants as compared to kainate alone. Similar results were obtained using NMDA rather than kainate. This was a surprise, because these carnosine derivatives were almost equally effective in preventing the rise in ROS (see Table 1). This observation indicates that the protective action of carnosine against excitotoxicity cannot be explained by its ability to scavenge ROS. Nor is it due to metal ion chelating, since histidine (10 mM in the preincubation medium) which is a more effective chelator than carnosine, had no effect on either NMDA- or kainate-induced DCF fluorescence (data not shown).

DISCUSSION

These experiments have shown that carnosine and its homologs, homocarnosine and N-acetylcarnosine, are all effective in reducing ROS in cerebellar granule cell neurons, as has been demonstrated in other biological systems.[14,16,19] However, only carnosine was effective in reducing the apoptotic cell death induced by application of the excitatory amino acid agonists, NMDA and kainate. These observations support the conclusion that the actions of ROS do not contribute directly to cell death in this preparation to the degree that has been suggested by previous studies.[15,23] Satoh *et al.*[42] reported that nerve growth factor and Bcl-2 protect against hydrogen peroxide-induced apoptosis in PC12 cells, but

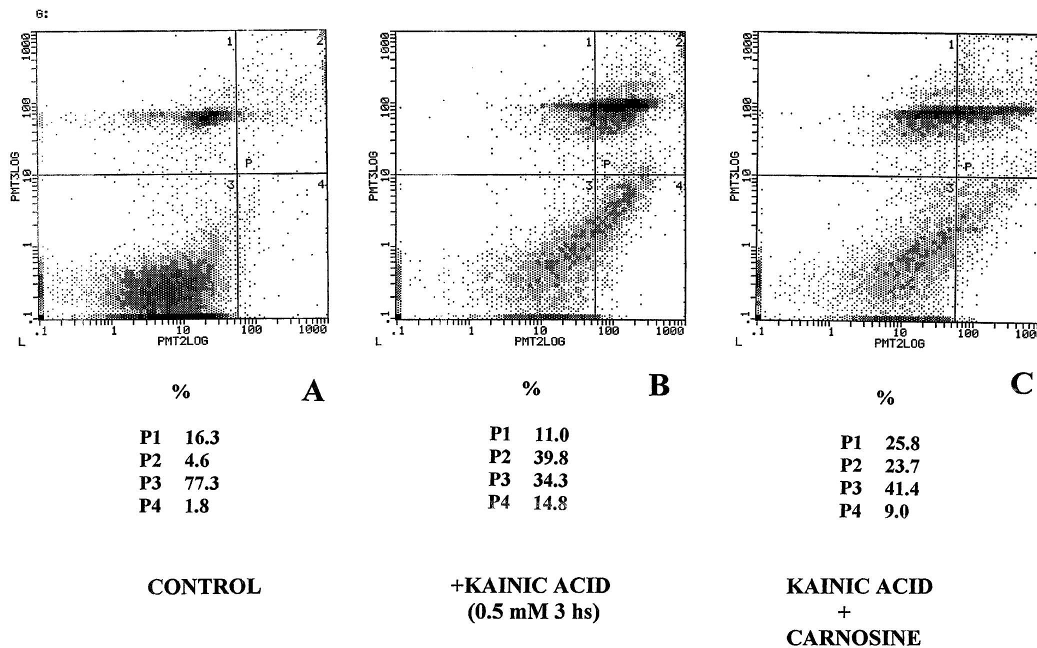


Fig. 4. Effect of carnosine on cell death induced by prolonged incubation in kainate. PI fluorescence is indicated on the *Y*-axis, while Annexin-V fluorescence is on the *X*-axis. (A) Control. (B) After 3 h exposure to 0.5 mM kainate. (C) The same as B but with the neurons preincubated with carnosine. The percentage of cells in each quadrant is shown under the pictures.

Table 2. Distribution of propidium iodide mean fluorescence values among several sub-populations of rat cerebellum granule cells after exposure to *N*-methyl-D-aspartate or kainate

Conditions	Peak I	Peak II	Peak III
Control	66.7 ± 2.1	16.7 ± 1.5	16.7 ± 1.2
+ Carnosine	82.9 ± 0.8**	–	17.1 ± 1.9
NMDA (2 mM, 60 min)	47.0 ± 12.3*	28.0 ± 4.6*	24.0 ± 2.6*
+ Carnosine	76.3 ± 12.4†	4.3 ± 3.5††	19.3 ± 4.9
Kainate (2 mM, 60 min)	43.0 ± 14.1*	34.8 ± 10.8**	22.0 ± 3.6
+ Carnosine	79.6 ± 9.8†	3.0 ± 2.0††	17.8 ± 5.8

Statistically significant difference from control is noted with $*P < 0.5$ or $**P < 0.01$ and from the values in the presence of NMDA or kainate with $†P < 0.05$ or $^{††}P < 0.01$.

Table 3. Effects of carnosine and related compounds on distribution of cerebellar granule cells among several populations labeled with propidium iodide and/or Annexin-V after exposure to kainate (0.5 mM, 3 h)

Conditions	An-V neg	An-V pos	An-V neg	An-V pos
	PI neg	PI neg	PI pos	PI pos
Quadrants	P3	P4	P1	P2
Control	79 ± 3	5 ± 1	6 ± 2	10 ± 2
Kainate	40 ± 3**	37 ± 4**	3 ± 1	20 ± 1*
+ Carnosine	67 ± 2††	3 ± 1††	23 ± 3††	7 ± 4†
+ N-acetylcarnosine	36 ± 4**	38 ± 4**	3 ± 1	23 ± 2*
+ Homocarnosine	37 ± 2**	40 ± 3**	4 ± 1	20 ± 3*

Statistically significant difference from control is noted with $*P < 0.05$ or $**P < 0.01$; statistically significant difference from kainate is noted with $†P < 0.05$ or $††P < 0.01$. Distribution of neurons between different quadrants as in Fig. 4. An-V, Annexin-V.

have no effect on ROS generation. This is consistent with our conclusion that agents that protect cells from death by apoptosis are dependent upon factors other than just ROS.

There is considerable evidence that carnosine and related compounds have free radical scavenging activity.[5,16,20,40] There is also considerable evidence that carnosine is protective against cell damage. Carnosine protects cell morphology in rat brain endothelial cells during nutritional insult,[18] protects against senescence of cultured fibroblasts[29] and delays loss of electrical excitability of neurons subjected to ischemia.[8] All of these actions have been attributed to radical scavenging activities.

Generation of ROS has been widely viewed as being a common pathway to cell death from a variety of injurious agents.[1,11] ROS have been specifically implicated in excitotoxicity (cell death induced by excessive amounts of the endogenous excitatory amino acid neurotransmitters, glutamate and aspartate) and the actions of exogenous agonists that act at their receptors, including NMDA and kainate.[34,44] However, others[6,10] have cautioned that while many injurious stimuli promote generation of ROS, the cell death that follows may not be as tightly coupled to ROS generation as many have thought.

If the neuroprotective actions of carnosine are not due to scavenging of ROS, there must be some other mechanism of action. One likely possibility is non-enzymatic glycosylation (glycation). Glycation is the Maillard reaction which consists of a cross-linking of protein amino groups with sugar aldehyde or keto groups to yield a heterogeneous group of irreversible adducts known as advanced glycation end products (AGEs). This process is known to occur in brain upon oxidative stress and in diseases like diabetes where sugars are elevated, and has been suggested to be a contributing factor in Alzheimer's disease secondary to glycation of tau protein.[52] AGEs also accumulate with normal aging.[53] The AGEs are known to be a source of ROS, and also activate receptors for a range of biologically important responses.[24]

In several models carnosine has been demonstrated to be an anti-glycating agent,[13,17,22,31] almost certainly through being itself very readily glycosylated. The preferred glycation

sites in proteins are the ϵ-amino groups of lysine residues, especially when in close proximity to histidine residues.[45] The structure of carnosine is very similar to Lys-His. Homocarnosine and N-acetylcarnosine have been demonstrated to be much less effective than carnosine as anti-glycating agent.[7]

CONCLUSION

Our hypothesis, to be tested further, is that carnosine protects neurons against excitotoxic damage because of its anti-glycating activity, not its ability to scavenge ROS. Whether this is the correct explanation or not, our observations make clear that carnosine has at least two different mechanisms of action, and suggest that the role of ROS in neuronal cell death following excitotoxic injury is not always as important as previously thought.

Acknowledgements—The work was supported by the Fullbright Foundation (U.S.A.), the Russian Foundation for Fundamental Research, Grant # 96-04-49078 and NIH ES04913 to DOC.

REFERENCES

1. Ames B. N., Shigenaga M. K. and Hagen T. M. (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. natn. Acad. Sci. U.S.A.* **90,** 7915–7922.
2. Aruoma O. I., Laughton M. J. and Halliwell B. (1989) Carnosine, homocarnosine and anserine: could they act as antioxidants *in vivo*? *Biochem. J.* **264,** 863–869.
3. Bate-Smith E. C. (1938) The buffering of muscle in rigor; protein, phosphate and carnosine. *J. Physiol., Lond.* **92,** 336–343.
4. Berkowitcz D. A., Tromley P. Q. and Shepherd G. M. (1994) Evidence for glutamate as the olfactory receptor cell neurotransmitter. *J. Neurophysiol.* **71,** 2557–2561.
5. Boldyrev A., Dupin A., Bunin A., Babizhaev M. and Severin S. (1987) The anti-oxidative properties of carnosine, a natural histidine-containing dipeptide. *Biochem. Int.* **15,** 1105–1113.
6. Boldyrev A. A., Lawrence D. A. and Carpenter D. O. (1998) Effect of carnosine and its natural derivatives on apoptosis of neurons induced by excitotoxic compounds. In *Peptide Science B Present and Future* (ed. Shimonishi Y.), pp. 424–426. Kluwer Acad., Manchester, U.K.
7. Boldyrev A. A., Carpenter D., Kovalenko Z. S., Kuleva N. V., Lawrence D. and Song R. (1999) Carnosine protects neurons from excitotoxic effects of NMDA and kainate. In *Maturation Phenomenon in Cerebral Ischemia* (ed. Ito U.), p. 529. Springer–Verlag, Berlin, Heidelberg.
8. Boldyrev A. A., Stvolinsky S. L., Tyulina O. V., Koshelev V. B., Hori N. and Carpenter D. O. (1997) Biochemical and physiological evidence that carnosine is an endogenous neuroprotector against free radicals. *Cell. molec. Neurobiol.* **17,** 259–271.
9. Carpenter D., Stoner C. R. T. and Lawrence D. A. (1997) Flow cytometric measurements on neuronal death triggered by PCBs. *NeuroToxicology* **18,** 507–514.
10. Ciani E., Gronend L., Voltattorni M., Rolseth V., Contestabile A. and Paulsen R. E. (1996) Inhibition of free radical production or free radical scavenging protects from the excitotoxic cell death mediated by glutamate in cultures of cerebellar granule neurons. *Brain Res.* **728,** 1–6.
11. Coyle J. T. and Puttfarcken P. (1993) Oxidative stress, glutamate and neurodegenerative disorders. *Science* **262,** 689–694.
12. Darzynkiewicz Z., Juan G., Xun L., Gorczica W., Murakami T. and Traganos F. (1997) Cytometry in cell neurobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* **27,** 1–20.
13. Degunie V., Menasche M., Ferrari P., Fraisse L., Poulinquen Y. and Robert L. (1998) Free radical depolymerization of hyaluronan by Maillard reaction products. Role in liquefaction of aging vitreous. *Int. J. biol. Macromolec.* **22,** 17–22.
14. Evsitgneeva R. P., Zheltukhina G. A., Rozkova E. A. and Grigorjev D. N. (1996) Interrelation between structure of N-acyl substituting group, conformation and antioxidant properties of carnosine, carcinine and their structural analogs. *Proc. Akad. Sci. Russia* **349,** 129–132.
15. Gunasekar P. G., Kanthasamy A. G., Borowitz J. L. and Ison G. E. (1995) NMDA receptor activation produces concurrent generation of nitric oxide and reactive oxygen species: implication for cell death. *J. Neurochem.* **65,** 2016–2021.
16. Hartman P. E., Hartman Z. and Ault K. T. (1990) Scavenging of singlet molecular oxygen by imidazole compounds: high and sustained activities of carboxy terminal histidine dipeptides and exceptional activity of imidazole-4-acetic acid. *Photochem. Photobiol.* **51,** 59–66.
17. Hipkiss A. R. (1998) Carnosine, a protective, anti-aging agent? *Int. J. Biochem. Cell Biol.* **30,** 863–868.
18. Kantha S. S., Wada S.-I., Tanaka H., Takeuchi M., Watabe S. and Ochi H. (1996) Carnosine sustains the retention of cell morphology in continuous fibroblast culture subjected to nutritional insult. *Biochem. biophys. Res. Commun.* **223,** 278–282.
19. Klebanov G. I., Teselkin Yu. O., Babenkova I. V., Popov I. N., Levin G., Tyulina O. V., Boldyrev A. A. and Vladimirov Yu. A. (1997) Evidence for a direct interaction of superoxide anion radical with carnosine. *Biochem. molec. Biol. Int.* **43,** 99–106.
20. Kohen R., Yamamoto Y., Cundy K. C. and Ames B. N. (1988) Antioxidant activity of carnosine, homocarnosine and anserine present in muscle and brain. *Proc. natn. Acad. Sci. U.S.A.* **85,** 3175–3179.
21. Kuba T., Enokido Y., Yamada M., Oka T., Uchiyama Y. and Hatanaka H. (1996) Oxygen-induced apopotosis in PC12 cells with special reference to the role of Bcl-2. *Brain Res.* **733,** 175–183.
22. Kuleva N. V. and Kovalenko Z. S. (1997) Changes in the functional properties of actin by its glycation *in vitro*. *Biochemistry, Moscow* **62,** 1119–1123.
23. LeBel C. P. and Bondy S. C. (1991) Persistent protein damage reduced oxygen radical formation in the aging rat brain. *Int. J. dev. Neurosci.* **9,** 139–146.
24. Li-Y M., Mitsuhashi T., Wojciechowicz D., Shimizu N., Li J., Stitt A., He C., Banerjee D. and Vlassara H. (1996) Molecular identity and cellular distribution of advanced glycation end product receptors: relationship of p60 to OST-48 and p90 to 80K-H membrane proteins. *Proc. natn. Acad. Sci. U.S.A.* **93,** 11,047–11,052.
25. MacLeod N. K. and Straughan D. W. (1979) Responses of olfactory bulb neurones to the dipeptide carnosine. *Expl Brain Res.* **34,** 183–188.
26. Margolis F. L. (1980) Carnosine: an olfactory neuropeptide. In *The Role of Peptides in Neuronal Function* (eds Barker J. L. and Smith T. G. Jr), pp. 545–572. Marcel Dekker, New York.
27. Margolis F. L. (1974) Carnosine in the primary olfactory pathway. *Science* **184,** 909–911.
28. Markesbery W. R. (1997) Oxidative stress hypothesis in Alzheimer's disease. *Free Rad. Biol. Med.* **23,** 134–147.
29. McFarland G. A. and Holliday R. (1994) Retardation of the senescence of cultured human diploid fibroblasts by carnosine. *Expl Cell Res.* **212,** 167–175.
30. McGowan A. J., Fernandes R. S., Samali A. and Cotter T. G. (1996) Anti-oxidants and apoptosis. *Biochem. Soc. Trans.* **24,** 229–233.
31. Munch G., Mayer S., Michaelis J., Hipkiss A. R., Riederer P., Muller R., Neumann A., Schinzel R. and Cunningham A. M. (1997) Influence of advanced glycation end-products and AGE-inhibitors on nucleation-dependent polymerization of beta-amyloid peptide. *Biochim. Biophys. Acta* **1369,** 17–29.
32. Nadi N. S., Hirsch J. D. and Margolis F. L. (1980) Laminar distribution of putative neurotransmitter amino acids and ligand binding sites in the dog olfactory bulb. *J. Neurochem.* **34,** 138–146.
33. Oyama Y., Carpenter D. O., Chikahisa L. and Okazaki E. (1996) Flow-cytometric estimation on glutamate- and kainate-induced increases in intracellular Ca^{2+} of brain neurons: a technical report. *Brain Res.* **728,** 121–124.
34. Patel M., Day B. J., Crapo J. D., Fridovich I. and McNamara J. O. (1996) Requirement for superoxide in excitotoxic cell death. *Neuron* **16,** 345–355.
35. Qin Z.-H., Wang Y. and Chase T. N. (1996) Stimulation of *N*-methyl-D-aspartate receptors induces apoptosis in rat brain. *Brain Res.* **725,** 166–176.

36. Reutelingsperger C. P. and van Heerde W. L. (1997) Annexin V, the regulator of phosphatidylserine-catalyzed inflammation and coagulation during apoptosis. *Cell. molec. Life Sci.* **53,** 527–532.
37. Reynolds I. J. and Hastings T. G. (1995) Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. *J. Neurosci.* **15,** 3318–3327.
38. Richter C. (1993) Pro-oxidants and mitochondrial Ca^{2+}: their relationship to apoptosis and oncogenesis. *Fedn Eur. biochem. Socs Lett.* **125,** 104–107.
39. Rothman S. M. (1985) The neurotoxicity of excitatory amino acids is produced by passive chloride influx. *J. Neurosci.* **5,** 1483–1489.
40. Salim-Hanna M., Lissi E. and Videla L. A. (1991) Free radical scavenging activity of carnosine. *Free Rad. Res. Commun.* **14,** 263–270.
41. Sassoe-Pognetto M. S., Catino D., Panzanelli P., Verdun-di-Cantogno L., Giustetto M., Margolis F. L., DeBiasi S. and Fasolo A. (1993) Presynaptic co-localization of carnosine and glutamate in olfactory neurones. *NeuroReport* **5,** 7–10.
42. Satoh T., Numakawa T., Abiru Y., Yamagata T., Ishikawa Y., Enokido Y. and Hatanaka H. (1998) Production of reactive oxygen species and release of L-glutamate during superoxide anion-induced cell death of cerebellar granule neurons. *J. Neurochem.* **70,** 316–324.
43. Schreiber S. J., Megow D., Raupach A., Victorov I. V. and Dirnagl U. (1995) Age-related changes in oxygen free radical production in the rat brain slice after hypoxia: on-line measurement using enhanced chemiluminescence. *Brain Res.* **703,** 227–230.
44. Schulz J. B., Henshaw D. R., Siwek D., Jenkins B. G., Ferrante R. J., Cipolloni P. B., Kowall N. W., Rosen B. R. and Beal M. F. (1995) Involvement of free radicals in excitotoxicity *in vivo*. *J. Neurochem.* **64,** 2239–2247.
45. Shilton B. H. and Walton D. J. (1991) Sites of glycation of human and horse liver alcohol dehydrogenase *in vivo*. *J. biol. Chem.* **266,** 5587–5592.
46. Simonian N. A., Getz R. L., Leveque J. C., Konrad C. and Coyle J. T. (1996) Kainate induces apoptosis in neurons. *Neuroscience* **74,** 675–683.
47. Sobue D., Konishi H. and Nakajama T. (1975) Isolation and identification of N-acetyl-homocarnosine and N-acetyl-carnosine from brain and muscle. *J. Neurochem.* **24,** 1261–1263.
48. Van-Engeland M., Nieland L. J. W., Ramaekers F. C. S., Schutte B. and Reutelingsperger C. (1998) Annexin-V affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* **31,** 1–9.
49. Vladimirov Yu.A. (1996) Studies of the antioxidant activity by measuring chemiluminescence kinetics. In *Proceedings of the International Symposium on Natural Antioxidants. Molecular Mechanisms and Health Effects* (eds Parcker L., Traber M. G. and Xin W.), pp. 125–144. AOCS Press, Champaign, Illinois.
50. Wood K. A. and Youle R. J. (1995) The role of free radicals and p53 in neuron apoptosis *in vivo*. *J. Neurosci.* **15,** 5851–5857.
51. Yamashita T., Shimada S., Guo W., Sato K., Kohmura E., Hayakawa T., Takagi T. and Tohyma M. (1997) Cloning and functional expression of a brain peptide/histidine transporter. *J. biol. Chem.* **272,** 10,211–10,295.
52. Yan S.-D., Chen X., Schmidt A.-M., Brett J., Godman G., Zou Y.-S., Scott C. W., Caputo C., Frappier T., Smith M. A., Perry G., Yen S.-H. and Stern D. (1994) Glycated tau protein in Alzheimer disease: a mechanism for induction of oxidant stress. *Proc. natn. Acad. Sci. U.S.A.* **91,** 7787–7791.
53. Yan S.-D., Schmidt A.-M., Anderson G. M., Brett J., Mora R., Nowygrod R. and Stern D. (1994) Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptor/binding proteins. *J. biol. Chem.* **269,** 9889–9897.

(*Accepted* 28 *April* 1999)