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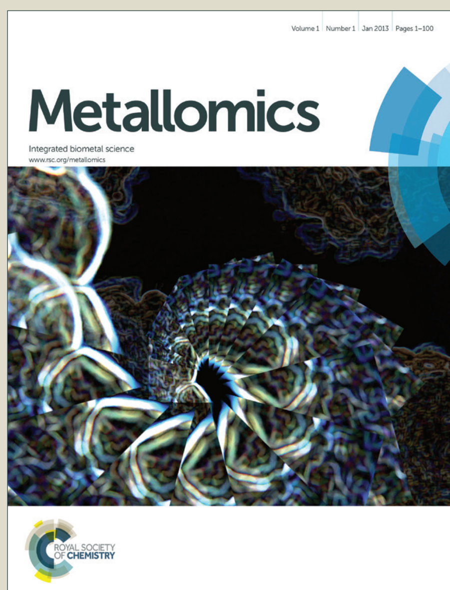

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ARTICLE

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Protective activity of carnosine and anserine against zinc–induced neurotoxicity: a possible treatment for vascular dementia

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Carnosine (β–alanyl–L–histidine) is a small dipeptide with numerous beneficial effects, including the maintenance of the acid–base balance, antioxidant properties, chelating agent, anti–crosslinking, and anti–glycation activities. High levels of carnosine and its analogue anserine (1–methyl carnosine) are found in skeletal muscle and the brain. Zinc (Zn)–induced neurotoxicity plays a crucial role in the pathogenesis of vascular dementia (VD), and carnosine inhibits Zn–induced neuronal death. Here, the protective activity of carnosine against Zn–induced neurotoxicity and its molecular mechanisms such as cellular Zn influx and Zn–induced gene expression were investigated using immortalised hypothalamic neurons (GT1–7 cells). Carnosine and anserine protected against Zn–induced neurotoxicity not by preventing increases in intracellular Zn^{2+} but by participating in the regulation of the endoplasmic reticulum (ER) stress pathway and the activity–regulated cytoskeletal protein (Arc). Accordingly, carnosine and anserine protected against neurotoxicity induced by ER–stress inducers thapsigargin and tunicamycin. Hence, carnosine and anserine are expected to have future therapeutic potential for VD and other neurodegenerative diseases.

Introduction

Carnosine, a dipeptide composed of β–alanine and L–histidine (His), is synthesised by carnosine synthase in an ATP–consuming reaction. Together with its methylated analogue anserine (1–methyl carnosine), it is found in various vertebrates such as fish, birds, and mammals.[1] In mammals, carnosine and anserine are mainly located in skeletal muscle and nervous tissues.[1,2] In the brain, carnosine accumulates in neurons of the olfactory bulbs and in glial cells elsewhere. Numerous beneficial characteristics of carnosine have been reported, including the maintenance of the pH balance,[3] anti–glycation,[4] and activities as an antioxidant,[5] hydroxyl radical scavenger,[6] and chelator of metal ions.[7,8] As these properties are related to ageing, and olfactory bulbs are gateways to the external environment, carnosine is assumed to be an endogenous anti–ageing or neuroprotective agent.

We have previously investigated the molecular mechanism of zinc–induced neuronal death. Accumulating evidence suggests that zinc (Zn) is central to ischemia–induced neuronal death and, eventually, to the pathogenesis of vascular dementia (VD).[9] In ischemic conditions, a considerable amount of Zn (up to 300 μM) is co–released with glutamate into synaptic clefts by membrane depolarisation. After transient global ischemia, the movement of chelatable Zn from presynaptic terminals into postsynaptic neuronal cell bodies occurs in vulnerable neurons in the CA1 or CA3 regions of the hippocampus,[10] and this movement can increase the likelihood of an infarct.[11] Zn causes apoptotic death in primary cultured cortical neurons[12] and PC12 cells, a pheochromocytoma cell line.[13] A membrane–impermeable chelating agent such as calcium EDTA (Ca–EDTA) binds to Zn^{2+}, blocks Zn influx into neuronal cell bodies, protects hippocampal neurons after transient global ischemia, and reduces infarct volume.[14] These results strongly suggest that Zn plays a key role in delayed neuronal death after transient global ischemia, a process that is potentially involved in the pathogenesis of VD. [9] Thus, we hypothesised that substances that protect against Zn–induced neuronal death could be potential candidates for preventing or treating neurodegeneration following ischemia, ultimately providing clues for novel VD treatments.[15,16]

Recently, we found that immortalised hypothalamic neurons (GT1–7 cells) are more vulnerable to Zn than other neuronal cells.[17,18] Zn causes apoptotic death of GT1–7 cells in a dose– and time–dependent manner. Mellon et al. originally developed GT1–7 cells by genetically targeting tumorigenesis in mouse hypothalamic neurons.[19] The cells exhibit neuronal characteristics such as the extension of neurites and the secretion or expression of several neuron–specific proteins or receptors. In addition, GT1–7 cells either lack or express low levels of ionotropic glutamate receptors and do not show glutamate toxicity.[20] These properties make the GT1–7 cell line an excellent model system for investigating Zn–induced neurotoxicity. We used GT1–7 cells to screen for substances that

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protect neurons against Zn, based on the idea that such substances might be potential candidates for VD therapeutics.[21–24]

Using this screening system, we examined the potential protective activity of carnosine against Zn–induced neuronal death. Further, we investigated the molecular mechanism of this protective activity by evaluating Zn influx and Zn–induced intracellular factor expression in GT1–7 cells. We previously demonstrated that Zn exposure upregulates various genes, including metal–binding proteins, endoplasmic reticulum (ER)–stress–related genes, and Ca^{2+} homeostasis–related genes.[25] As the disruption of Ca^{2+} homeostasis may be involved in Zn–induced neurotoxicity, we focused on the implications of these factors in the protective activity of carnosine.

Results and discussion

Protective activity of carnosine and anserine against Zn–induced neurotoxicity

Previously, we showed that His and its various analogues exhibit protective activity against Zn–induced neuronal death.[25] Here, GT1–7 cells were simultaneously treated with Zn and carnosine, His, or anserine. Carnosine and anserine inhibited Zn–induced neurotoxicity in a dose–dependent manner. It is interesting to note that olfactory bulb neurons are less sensitive to damage after ischemia than are hippocampal neurons in spite of an equivalent accumulation of Zn.[26] Carnosine accumulates in neurons of the olfactory bulbs.[1] These observations suggested that carnosine and anserine may play protective roles in Zn–induced neurodegeneration in the olfactory bulb.

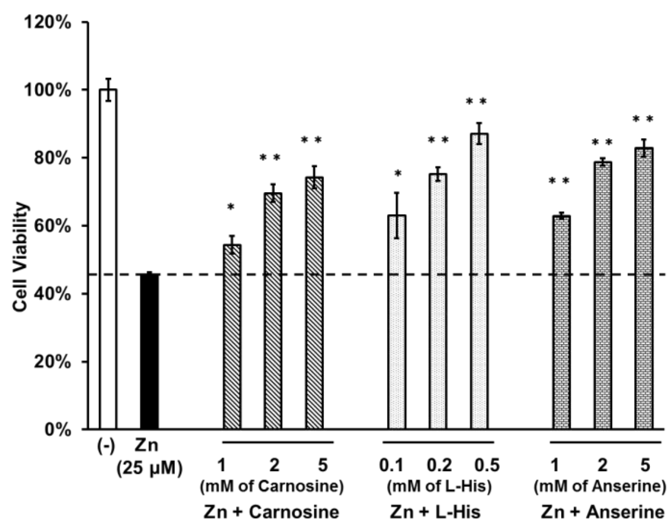

Fig. 1 Protective activity of carnosine, His, and anserine against Zn–induced neurotoxicity in GT1–7 cells. Twenty–four hours after $ZnCl_2$ (25 μM) administration to GT1–7 cells, cell viability was compared among groups treated with various concentrations of carnosine, His, or anserine. Data are presented as means ± S.E.M., $n = 6$. * $p < 0.05$, ** $p < 0.01$.

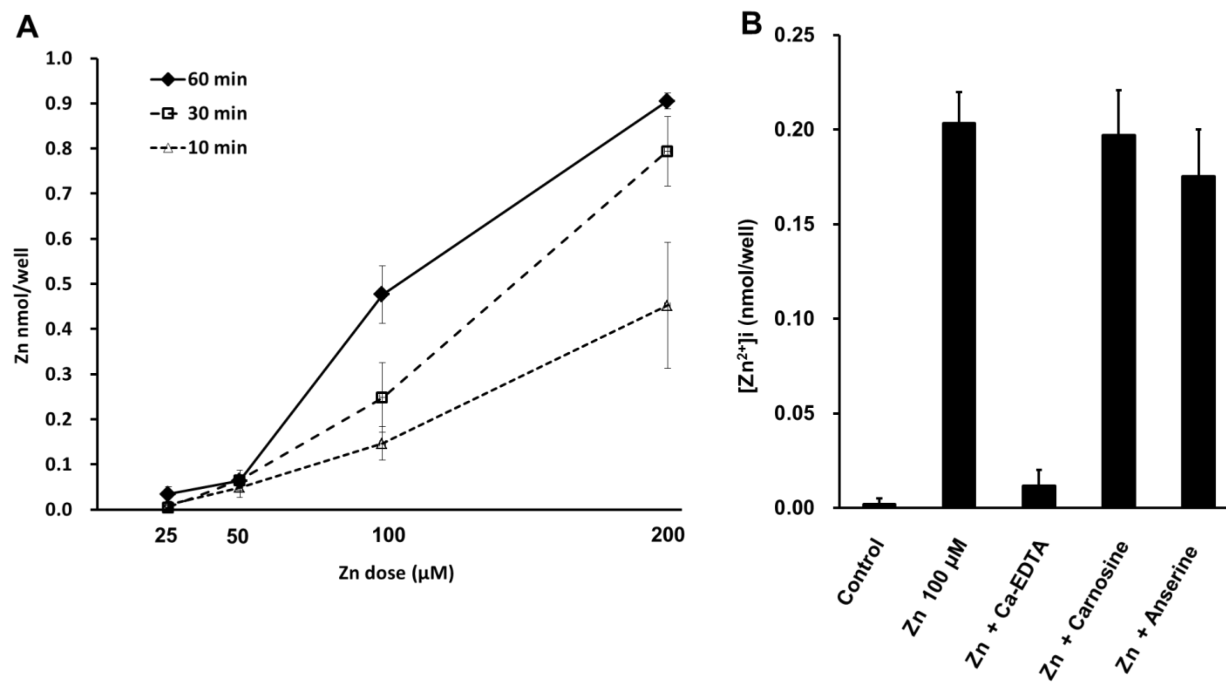

Fig. 2 Effects of carnosine and anserine on Zn influx into GT1–7 cells. A, Cells were treated with various concentrations of $ZnCl_2$, and after 10 (triangle), 30 (square), or 60 (rhombus) min $[Zn^{2+}]_i$ was measured as described in *Materials and Methods*. B, GT1–7 cells were treated with Ca–EDTA (0.2 mM), carnosine (2.0 mM), or anserine (2.0 mM) and 100 μM of $ZnCl_2$ for 30 min, and $[Zn^{2+}]_i$ was measured as described in *Materials and Methods*. Data are presented as means ± S.E.M., $n = 3$.

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Protective activity of carnosine and anserine was weaker than His. To obtain an equivalent protective effect of His, 10-fold concentration of carnosine or anserine was required (Fig. 1). Our previous study hinted at the importance of a free NH_2 group at the N–terminus of His for the protective activity against Zn–induced neuronal death, because compounds such as His–amide and His–Ala exhibited similar protective activity as His, while compounds lacking this functional group such as histamine, imidazole and acetyl–His were not effective.[25] These findings indicated that the weaker protective activity of carnosine and anserine is caused by the lack of this functional group. Furthermore, we have already revealed that carnosine attenuated Zn-induced death of primary cultured neurons of rat hippocampus.

Effects of carnosine and anserine on Zn influx into GT1–7 cells

To investigate the molecular mechanism of the protective activity of carnosine, we analysed Zn translocation as the primary event in the signalling pathway mediating Zn–induced neuronal death.[27] As the His residue of carnosine can chelate cations[7,8] such as Zn^{2+}, we assumed that carnosine inhibits Zn influx into GT1–7 cells by binding to Zn in the culture medium. However, we have previously shown that His does not influence Zn influx into GT1–7 cells, despite its chelating activity.[25] First, we analysed intracellular Zn^{2+} levels ($[Zn^{2+}]_i$) in Zn–treated GT1–7 cells in a dose– and time–dependent manner. Second, we evaluated the effects of carnosine and anserine on $[Zn^{2+}]_i$ at 30 min after treatment with 100 μM Zn, a period short enough to not affect cell viability (data not shown). Whereas treatment with Ca-EDTA, a membrane impermeable chelator, decreased $[Zn^{2+}]_i$ in GT1–7 cells, neither carnosine nor anserine inhibited Zn influx into GT1–7 cells (Fig. 2B), comparable to His.[25]

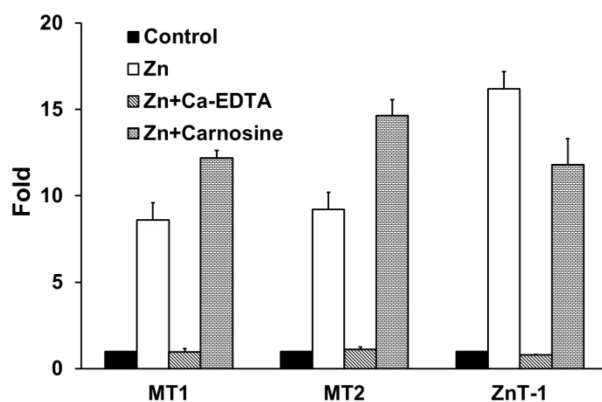

Fig. 3 Effects of carnosine on Zn–induced expression of metal–binding proteins. GT1–7 cells were treated with 50 μM $ZnCl_2$ for 6 h in the absence or presence of Ca–EDTA (0.5 mM) or carnosine (5.0 mM). Expression of *MT1*, *MT2*, and *ZnT–1* was analysed by RT–PCR, and gene expression levels were normalised with β–actin. Data are presented as means ± S.E.M., $n = 3$. ** $p < 0.01$.

Furthermore, the expression of *metallothionein–1* (*MT1*), *metallothionein–2* (*MT2*), and *zinc transporter–1* (*ZnT–1*) genes in GT1–7 cells was analysed. These proteins regulate $[Zn^{2+}]_i$, MT1 and MT2 playing roles in the detoxification of heavy metals, while Zn transporters control Zn homeostasis, facilitating Zn influx during times of deficiency and efflux during times of Zn excess. The mRNA levels of *MT1*, *MT2*, and *ZnT–1* were significantly increased after 6 h of Zn treatment, while Ca–EDTA abolished this induction (Fig. 3), probably chelating Zn^{2+} outside the cells and thereby inhibiting an increase of $[Zn^{2+}]_i$. However, carnosine did not significantly inhibit the Zn–induced upregulation of these genes, indicating that chelation of Zn^{2+} ions by carnosine and anserine does not cause the protective activity against Zn–induced neurotoxicity as in the case of His.

Carnosine participates in the endoplasmic reticulum (ER)–stress pathway in Zn–induced neurotoxicity

Based on the involvement of Ca^{2+} homeostasis in Zn–induced neurotoxicity[18], we focused on the ER–stress pathway. It is widely accepted that the ER regulates the intracellular calcium level ($[Ca^{2+}]_i$), and that ER–stress causes apoptotic cell death via the accumulation of misfolded or unfolded proteins.[28] ER–stress has been implicated in the pathogenesis of various neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), prion disease, and ischemia–induced neurodegeneration.[29,30] As shown in Fig. 4A, Zn–induced neuronal death upregulated several genes, including ER stress–related genes such as growth–arrest DNA damage (*GADD*)*34*, *GADD45*, and *p8*, and the Ca^{2+}–related activity–regulated cytoskeletal protein (*Arc*). *GADD34* and *GADD45*, encoding the sensor proteins of ER–stress, are induced by DNA damage and have been implicated in DNA repair and tumorigenesis.[31] Whereas *p8* mRNA is induced in response to diverse stresses and may be involved in tumorigenesis,[32] *Arc* encodes a dendrite protein that plays crucial roles in synaptic plasticity and memory consolidation. Arc expression is induced by the increased neuronal activity that ensues in response to learning and by the brain–derived neurotrophic factor (BDNF).[33] Thus, we previously investigated the detailed characteristics of Zn-induced upregulation of these genes by RT-PCR. The expressions of these genes were significantly increased compared to the control groups, after exposure to Zn (Fig. 4A). The induction of *GADD34*, *GADD45* and *Arc* after ischemia was reported previously.[34-36] Taken together these findings and our finding about Zn-induced upregulation of *GADD34*, *GADD45*, *p8*, and *Arc* suggest the implication of some ER–stress–related or Arc–related pathways in ischchemia–induced neuronal death and the pathogenesis of VD.

Analogous to metal–related genes (Fig. 3), Ca–EDTA treatment abolished the Zn–induced upregulation of all these genes, while carnosine partially attenuated the effects of Zn treatment (Fig. 4A). Induction of *GADD34* and *Arc* was significantly decreased by carnosine treatment but *GADD45* and *p8* were not changed (Fig. 4A). Whereas His abolished the Zn–induced upregulation of *GADD45* and *p8* gens in addition to *GADD34* and *Arc*.[25] The protective effects of carnosine were partially different from His. Hence, we assume that the effects of carnosine on the ER–stress pathway in Zn–induced neurotoxicity are due to carnosine itself. Particularly, the expression of *Arc* was markedly increased by Zn treatment (22.3 ± 1.4 fold) and decreased to 3.4 ± 1.0 fold by carnosine. Further, we investigated the

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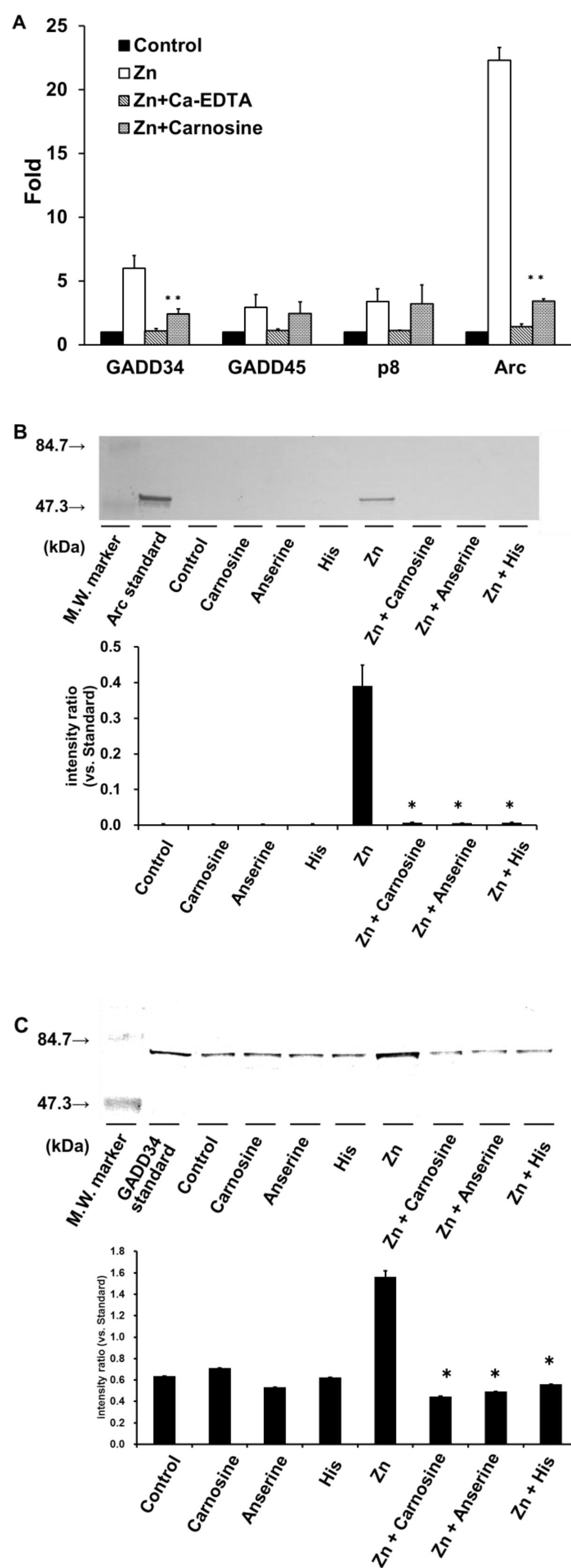

Fig. 4 Effects of carnosine on the expression of Zn–induced factors. A, Effects of carnosine on Zn–induced gene expression. GT1–7 cells were treated with 50 μM $ZnCl_2$ for 6 h in the absence or presence of Ca–EDTA (0.5 mM) or carnosine (5.0 mM). Expression of *GADD45*, *GADD34*, *p8*, and *Arc* was analysed by RT–PCR, and gene expression levels were normalised with β–actin. Data are presented as means ± S.E.M., $n = 3$. ** $p < 0.01$ vs. Zn group. Effects of carnosine and its analogues on Zn–induced Arc (B) and GADD34 (C) expression. These expressions in GT1–7 cells were assayed by western blotting using Arc– or GADD34 overexpressing lysates as a standard. GT1–7 cells were treated with 50 μM of $ZnCl_2$ for 6 h in the absence or presence of carnosine (5.0 mM), anserine (5.0 mM), or His (0.5 mM). The blot was probed with anti–Arc or anti-GADD34 antibody, respectively. Band intensities were analysed by ImageJ software. The values obtained by dividing intensities of the respective band by that of the standard band were shown in the graph. Data are presented as means ± S.E.M., $n = 3$. * $p < 0.05$ vs. Zn group.

effects of Zn, carnosine, and anserine treatment on Arc or GADD34 protein expressions in GT1–7 cells using western blot analysis. Expression of Arc protein was only detected in the lysate of Zn–treated GT1–7 cells, while carnosine and anserine decreased the induction of Arc protein to undetectable levels (Fig. 4B). Arc is related to synaptic plasticity and the exptression of brain-derived neurotrophic factor (BDNF).[33,37] It is widely accepted that the increase of $[Ca^{2+}]_i$ enhanced the expression of Arc. We have reported that Zn caused the increase of $[Ca^{2+}]_i$.[18] Therefore, it is possible that carnosine, anserine, and histidine blocked Zn-induced increase of $[Ca^{2+}]_i$ and inhibited arc expression or affects other unknown pathway. Further studies are necessary. As shown figure 4C, GADD34 expression in GT1-7 cells was significantly induced by Zn treatment (2.45 fold, $p < 0.05$ vs. control group).. Carnosine and anserine significantly decreased the Zn-induced GADD34 protein. Our results indicate that these dipeptides protect against Zn–induced neurotoxicity by blocking some ER–stress–related or Arc–related pathways. Further, we investigated the effects of carnosine on neuronal death induced by ER–stress inducers such as thapsigargin (TG) and tunicamycin (TC). TG is an inhibitor of the sarcoplasmic/ER calcium $(Ca)^{2+}$ ATPase that pumps Ca^{2+} into the ER, while tunicamycin inhibits N–linked protein glycosylation. Viability of GT1–7 cells treated with 10 nM TC or 0.5 nM TG was decreased to 45.4% ± 3.7% or 49.5% ± 8.3%, respectively (Fig. 5). Both carnosine and anserine exhibited protective activity against both TG– and TC–induced neurotoxicity in a dose–dependent manner. These findings support our hypothesis implying some ER–stress–related or Arc–related pathways in the protective effects of carnosine and anserine against Zn–induced neurotoxicity and the pathogenesis of VD. Furthermore, carnosine was found to be protective against other neurodegenerative diseases such as AD and prion diseases, *e.g.*, improving learning abilities of Alzheimer's model mice,[38] or attenuating the neurotoxicity of a prion protein fragment.[39] As ER–stress is underlying various neurodegenerative diseases[40] their protection against neurotoxicity by TG and TC makes carnosine and anserine candidates for ER–stress–related disease therapeutics. Alimentary–absorbed carnosine elevates the blood level of His via digestion in small intestinal cells.[41] Elevation of circulating levels of His raises the concentration of carnosine in skeletal muscle.[42,43] Therefore, dietary intake may accumulate

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carnosine in the body, including the brain, where it may be effective in the prevention of VD. In fact, it has been reported that supplementation of carnosine and anserine showed promising effects on cognitive functioning of elderly participants.[44] Carnosine and anserine may also be useful as drugs for the treatment of established VD.

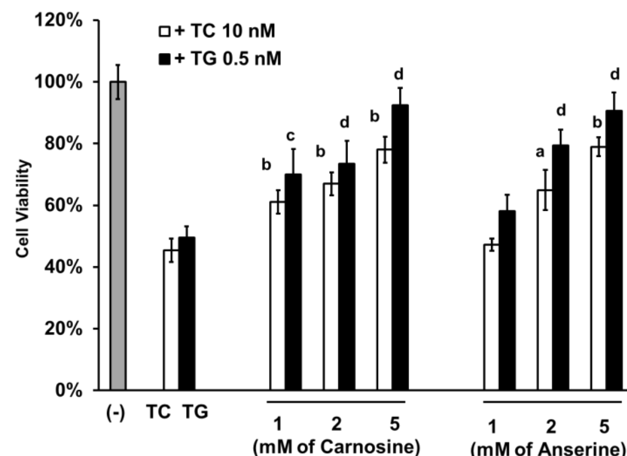

Fig. 5 Protective activitiy of carnosine and anserine against neuronal death induced by ER–stress inducers. GT1–7 cells were pretreated with 10 nM tunicamycin (TC: white bar) or 0.5 nM of thapsigargin (TG: black bar). After 24 h, viability of GT1–7 cells was compared with that of cells treated by co–administration of various concentrations of carnosine or anserine. Data are presented as means ± S.E.M., $n = 6$. a, $p < 0.05$; b, $p < 0.01$. vs. TC 10 nM. c, $p < 0.05$; d, $p < 0.01$. vs. TG 0.5 nM.

Experimental

Reagents

Analytical grade pharmacological reagents were used. TC, TG, His, anserine, and carnosine were purchased from Sigma Aldrich (St. Louis, MO, USA). $ZnCl_2$ was purchased from Nakalai Tesque (Kyoto, Japan).

Cell culture

GT1–7 cells (provided by Dr. R. Weiner, University of California San Francisco) were grown in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12) supplemented with 10% fetal bovine serum. After trypsin digestion, cells were resuspended in serum–free medium, distributed into culture dishes,[45] and cultured in a humidified incubator (7% CO_2) at 37°C.

Cell viability assay

Cell viability was assessed as previously described.[25] Briefly, dissociated GT1–7 cells were distributed into 96–well culture plates at a concentration of 5×10^4 cells per well in 200 µL culture medium. After 24 h incubation, cells were treated with various compounds prior to the addition of $ZnCl_2$ to the medium. After 24 h exposure, cell viability was quantified using a WST–based cell counting kit (Dojindo, Kumamoto, Japan). Absorbances of treated samples were measured against a blank control using an iMark microplate absorbance reader (Bio–Rad Laboratories, Hercules, CA, USA) at 450 nm and 620 nm detection and reference wavelength, respectively.

Quantification of intracellular Zn^{2+} levels

Concentrations of Zn^{2+} in GT1–7 cells were measured using a Metallo Assay Zinc LS kit (Metallogenics, Chiba, Japan) according to the manufacturer's instructions. GT1–7 cells were cultured in 96–well plates as described above, and Zn treatment conditions were optimised in a preliminary experiment as shown in Fig. 2A. To evaluate the effects of carnosine and anserine on $[Zn^{2+}]_i$, GT1–7 cells were exposed to 2 mM of each compound and 100 µM of $ZnCl_2$ for 30 min. Cells were washed twice with serum–free medium, lysed with 40 µL 10 mM CHAPS (Dojindo), 200 µL Metallo Assay Zinc LS kit were added, and absorbances of treated samples were measured against a blank control using an MTP–650 microplate reader (Corona Electric, Ibaragi, Japan) at 560 nm detection wavelength.

RT–PCR

Zn–induced gene expression was assessed as previously described.[25] Briefly, total RNA was extracted from GT1–7 cells grown in 12–well culture plates (1×10^6 cells per well) using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. First–strand cDNA was synthesised from 8 µL total RNA solution using a SuperScript III First–Strand Synthesis System for real–time quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR; Invitrogen, Carlsbad, CA, USA) priming with oligo(dT)20. Synthesised cDNA was diluted to 100 µL with distilled water. Real–time PCR was performed on the LightCycler (Roche Diagnostics, Mannheim, Germany) using Platinum SYBR Green qPCR SuperMix–UDG (Invitrogen, Carlsbad, CA, USA). The following PCR primers were used: *MT1*: 5'–GTA CCT TCT CCT CAC TTA CTC CG–3' (forward) and 5'–GTA TAG GAA GAC GCT GGG TTG G–3' (reverse); *ZnT–1*: 5'–GGC CAA CAC CAG CAA TTC CAA CG–3' (forward) and 5'–AAG GCA TTC ACG ACC ACG ATC ACG–3' (reverse); *GADD45*: 5'–ACA TCG TCC GGG TAT CAG GCA TG–3' (forward) and 5'–AGT GGG TCT CAG CGT TCC TCT AGG–3' (reverse); *GADD34*: 5'–CCT CTA AAA GCT CGG AAG GTA CAC–3' (forward) and 5'–TCG GAC TGT GGA AGA GAT GGG–3' (reverse); *p8*: 5'–ATG GCC ACC TTG CCA CCA AC G–3' (forward) and 5'–TCA GCG CCA GGC TTT TTT CCT TTC–3' (reverse); *Arc*: 5'–CTC ACT AGC TGC CTG CCA TTA G–3' (forward) and 5'–GAT GGA GGA ACC GCA ACA AGG–3' (reverse); *β–actin*: 5'–CGC ATC CTC TTC CTC CCT GG–3'(forward) and 5'–CCT AGA AGC ACT TGC GGT GCA C–3' (reverse).

Western blot

Zn–induced Arc and GADD34 expressions were assessed by western blot analysis. GT1–7 cells grown in 12–well culture plates (1×10^6 cells per well) were lysed with 200 µL 10 mM CHAPS. Arc–over–expressing lysate (Arc (h): 293T, sc-117312) or GADD-expressing U-937 Cell Lysate (sc-2239) were purchased from Santa Cruz

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Biotechnology (Santa Cruz, CA, USA) and used as an Arc- or GADD34-standard, respectively. Protein concentrations were measured using the bicinchoninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, IL, USA). Lysates were added to EzApply (ATTO, Tokyo, Japan), adjusted to a final protein concentration of 1 μg/μL or 0.1 μg/μL, and boiled at 95°C for 5 min. Ten microliters of each sample were applied to a gradient (10 – 20%) polyacrylamide gel (e–PAGEL(R) 10 – 20%; ATTO), electrophoresed at a constant current of 20 mA per gel, and proteins were transferred to polyvinylidene difluoride (PVDF) membranes using EzFastBlot (ATTO) at a constant current of 2 mA/cm^2 for 90 min. Membranes were blocked with EzBlock BSA (ATTO) at room temperature for 1 h, incubated with polyclonal rabbit anti-Arc (mouse) antibody (Santa Cruz) for 1 h and with HRP-conjugated goat anti–rabbit IgG (Abcam, Cambridge, MA, USA) for 1 h, and subsequently stained using EzWestBlue (ATTO). Band intensities were quantitated by using ImageJ open source software (version 1.48v for Windows), and the values obtained by dividing intensities of the respective band by that of the standard band were represented in the graph.

Statistical analyses

For statistical analyses, a two–tailed Student's *t*–test (StatView; SAS Institute, Cary, NC, USA) with a significance level of $p < 0.05$ was used.

Conclusions

It has been suggested that Zn is central to ischemia–induced neuronal death and to the pathogenesis of VD.[9] Here, protective activity of carnosine and anserine (the methylated analogue of carnosine) against Zn–induced neurotoxicity was demonstrated. Regarding the mechanism underlying their protective effects, neither carnosine nor anserine inhibited the influx of Zn into cell bodies. Instead, we hypothesised that they may inhibit several apoptotic pathways activated by Zn. Given the advantageous properties of carnosine and anserine (relatively nontoxic, heat–stable, and water–soluble), dietary supplementation with them may be an effective strategy for the prevention or treatment of neurodegenerative diseases such as ischemia–induced neuronal death and VD. Therefore, we have filed patents for carnosine (application No. 2006–145857, publication No. 2007–314467 in Japan) as a drug for the treatment of VD or for slowing the progress of cognitive decline after ischemia.[16]

While the physiological roles of carnosine in the olfactory bulb of the brain are still unclear, olfactory bulb neurons are less sensitive to damage after ischemia compared to hippocampal neurons, despite the accumulation of Zn. Furthermore, carnosine levels have been shown to vary during development,[46] and the content of carnosine in muscle is decreased in older animals.[47] Therefore, carnosine may play protective roles in Zn–induced neurodegeneration after ischemia in the olfactory bulb. It is plausible that carnosine may be transported into cell bodies, where it could inhibit several Zn–activated apoptotic pathways. In conclusion, we hope that our carnosine–focused approach may benefit the development of drugs for the treatment of VD and other neurodegenerative diseases.

Acknowledgements

This work was partially supported by a Grant–in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a Grant from the Cooperation of Innovative Technology and Advanced Research in Evolution Area (CITY AREA) of the Miyazaki Prefectural Industrial Support Foundation.

Notes and references

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1. A. A. Boldyrev, G. Aldini and W. Derave, *Physiol. Rev.*, 2013, **93**, 1803–1845.
2. A. R. Hipkiss, *Adv. Food. Nutr. Res.*, 2009, **57**, 87–154.
3. H. Abe, *Biochemistry (Mosc)*, 2000, **65**, 757–765.
4. Y. Chen and C. T. Ho, *J. Agric. Food. Chem.*, 2002, **50**, 2372–2376.
5. A. E. Gariballa and A. J. Sinclair, *Age. Ageing.*, 2000, **29**, 207–210.
6. J. H. Kang, *Bull. Korean. Chem. Soc.*, 2005, **26**, 1251–1254.
7. E. J. Baran, *Biochemistry (Mosc)*, 2000, **5**, 789–797.
8. P. Q. Trombley, M. S. Horning and L. J. Blakemore, *Biochemistry (Mosc)*, 2000, **65**, 807–816.
9. J. H. Weiss, S. L. Sensi and J. Y. Koh, *Trends Pharmacol. Sci.*, 2000, **21**, 395–401.
10. J. Y. Koh, S. W. Suh, B. J. Gwag, Y. Y. He, C. Y. Hsu and D. W. Choi, *Science*, 1996, **272**, 1013–1016.
11. J. M. Lee, G. J. Zipfel, K. H. Park, Y. Y. He, C. Y. Hsu and D. W. Choi, *Neuroscience*, 2002, **115**, 871–878.
12. J. Y. Koh and D. W. Choi, *Neuroscience*, 1994, **60**, 1049–1057.
13. A. H. Kim, C. T. Sheline, M. Tian, T. Higashi, R. J. McMahon, R. J. Cousins and D. W. Choi, *Brain Res.*, 2000, **886**, 99–107.
14. A. Calderone, T. Jover, T. Mashiko, K.M. Noh, H. Tanaka, M. V. Bennett and R. S. Zukin, *J. Neurosci.*, 2004, **24**, 9903–9913.
15. M. Kawahara, K. Konoha and Y. Sadakane, *Biomed. Res. Trace Elem.*, 2007, **18**, 26–34.
16. M. Kawahara, K. Konoha, T. Nagata and Y. Sadakane, *Recent. Pat. CNS Drug. Discov.*, 2007, **2**, 145–149.
17. M. Kawahara, M. Kato–Negishi and Y. Kuroda, *Cell Mol. Neurobiol.*, 2002, **22**, 87–93.
18. H. Koyama, K. Konoha, Y. Sadakane, S. Ohkawara and M. Kawahara, *J. Clin. Toxicol.*, 2012, **S3**, 002.

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19. P. L. Mellon, J. J. Windle, P. C. Goldsmith, C. A. Padula, J. L. Roberts and R. I. Weiner, *Neuron*, 1990, **5**, 1–10.
20. V. B. Mahesh, P. Zamorano, L. de Sevilla, D. Lewis and D. W. Brann, *Neuroendocrinology*, 1999, **69**, 397–407.
21. Y. Sadakane, K. Konoha and M. Kawahara, *Trace Nutr. Res.*, 2005, **22**, 73–79.
22. K. Konoha, Y. Sadakane and M. Kawahara, *Trace Nutr. Res.*, 2006, **23**, 56–62.
23. Y. Sadakane, K. Konoha, T. Nagata and M. Kawahara, *Trace Nutr. Res.*, 2007, **24**, 98–105.
24. Y. Sadakane, K. Konoha, T. Nagata and M. Kawahara, *Trace Nutr. Res.*, 2008, **25**, 41–45.
25. M. Kawahara, Y. Sadakane, H. Koyama, K. Konoha and S. Ohkawara, *Metallomics*, 2013, **5**, 453–460.
26. A. R. Hipkiss, *Rejuvenation Res.*, 2004, **7**, 253-255.
27. S. L. Sensi, L. M. Canzoniero, S. P. Yu, H. S. Ying, J. Y. Koh, G. A. Kerchner and D. W. Choi, *J. Neurosci.*, 1997, **17**, 9554–9564.
28. M. K. Brown and N. Naidoo, *Front. Physiol.*, 2012, **3**, 263.
29. E. Ferreiro, I. Baldeiras, I. L. Ferreira, R. O. Costa, A. C. Rego, C. F. Pereira and C. R. Oliveira, *Int. J. Cell Biol.*, 2012, DOI: 10.1155/2012/735206.
30. B. D. Roussel, A. J. Kruppa, E. Miranda, D. C. Crowther, D. A. Lomas and S. J. Marciniak, *Lancet Neurol.*, 2013, **12**, 105–118.
31. A. A. Moskalev, Z. Smit–McBride, M. V. Shaposhnikov, E. N. Plyusnina, A. Zhavoronkov, A. Budovsky, R. Tacutu and V. E. Fraifeld, *Ageing Res. Rev.*, 2012, **11**, 51–66.
32. N. van Prooyen, V. Andresen, H. Gold, I. Bialuk, C. Pise–Masison and G. Franchini, *Mol. Aspects Med.,* 2010, **31**, 333–343.
33. D. Panja and C. R. Bramham, *Neuropharmacology*, 2014, **76**, 664–76
34. S. Onoue, Y. Kumon, K. Igase, T. Ohnishi, and M. Sakanaka, *Brain Res. Mol. Brain Res.* 2005, **134**, 189–197.
35. H. Kunizuka, H. Kinouchi, S. Arai, K. Izaki, S. Mikawa, H. Kamii, T. Sugawara, A. Suzuki, K. Mizoi and T. Yoshimoto, *NeuroReport*, 1999, **10**, 1717–1722.
36. M. Rickhag, M. Teilum and T. Wieloch, *Brain Res.*, 2007, **1151**, 203–210.
37. E. Korb and S. Finkbeiner, *Trends Neurosci.* 2011, **34**, 591–598.
38. C. Corona, V. Frazzini, E. Silvestri, R. Lattanzio, R. La Sorda, M. Piantelli, L. M. Canzoniero, D. Ciavardelli, E. Rizzarelli and S. L. Sensi, *PLoS One*, 2011, **6**, e17971.
39. M. Kawahara, H. Koyama, T. Nagata and Y. Sadakane, *Metallomics*, 2011, **3**, 726–734.
40. D. Lindholm, H. Wootz and L. Korhonen, *Cell Death Differ.*, 2006, **13**, 385–92.
41. M. L. Gardner, K. M. Illingworth, J. Kelleher and D. Wood, *J. Physiol.*, 1991, **439**, 411–422.
42. L. M. Maynard, G. A. Boissonneault, C. K. Chow and G. G. Bruckner, *J. Nutr.*, 2001, **131**, 287–290.
43. N. Tamaki, A. Funatsuka, S. Fujimoto and T. Hama, *J. Nutr. Sci. Vitaminol. (Tokyo)*, 1984, **30**, 541–551.
44. D. Szcześniak, S. Budzeń, W. Kopeć and J. Rymaszewska, *Arch. Gerontol. Geriatr.*, 2014, **59**, 485–490.
45. M. Kawahara, N. Arispe, E. Rojas and Y. Kuroda, *J. Biol. Chem.*, 2000, **275**, 14077–14083.
46. S. de Marchis, C. P. Modena, P. Peretto, A. Migheli, F. L. Margolis and A. Fasolo, *Biochemistry (Mosc)*, 2000, **65**, 824–833.
47. H. J. Stuerenburg, *Biochemistry (Mosc)*, 2000, **65**, 862–865.

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