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Carnosine Protects GT1-7 Cells Against Zinc-induced Neurotoxicity: a Possible Candidate for Treatment for Vascular Type of Dementia

Keiko KONOHA, Yutaka SADAKANE, and Masahiro KAWAHARA*
Department of Analytical Chemistry, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare

Summary

Zinc is the second most abundant trace element in the brain. Considerable amount of zinc is co-released with glutamate to the synaptic cleft during the neuronal excitation. Recent studies have indicated that excess zinc has a causative role in delayed neuronal death after transient global ischemia ischemia. Therefore, it is possible that a substance which protects against zinc-induced neuronal death could be a candidate for the prevention or treatment of neurodegeneration after ischemia, and finally provide a clue to the drugs of vascular type of senile dementia. To explore such substances, we have developed a convenient and sensitive *in vitro* assay system using GT1-7 cells (immortalized hypothalamic neurons), and examined various substances including fish extracts. Among tested, we found that water-soluble, heat-stable extracts of eels significantly protected GT1-7 cells from zinc. The eel extract contained much amount of carnosine (β-alanyl histidine), and carnosine protected GT1-7 cells against zinc-induced neurotoxicity in a dose-dependent manner. Our results suggest that carnosine may become a candidate for a therapeutic target of the global ischemia.

Introduction

Zinc is abundantly present in the brain, particularly in the hippocampus, amygdala, cerebral cortex, and olfactory cortex[1]. A substantial amount of zinc (approximately 10 % or more) in the brain forms free zinc ions (Zn^{2+}) or is loosely bound and detectable by the staining using chelating reagents, while, other zinc firmly binds to metalloproteins or enzymes. The chelatable zinc is stored in the presynaptic vesicles of particular excitatory (glutamatergic) neurons, and is secreted from vesicles to synaptic clefts with excitatory neurotransmitter glutamate during the neuronal excitation[2]. Its concentration is estimated to be approximately 300 μM. Although its physiological role has not yet been defined precisely, zinc alters the behavior of various receptors or ion channels, and is believed to play crucial roles in the synaptic plasticity, learning and memory.

Nonetheless, recent studies have demonstrated that the disruption of zinc homeostasis causes severe damage to the central nervous system[3]. It is widely recognized that excess glutamate is released from nerve terminals to the synaptic clefts after transient global ischemia and triggers various pathways of apoptotic neuronal death[4]. The delayed neuronal death after ischemia is believed to have a causative role in subsequent cognitive dysfunction termed vascular type of dementia in elderly people[5]. Increasing evidence suggests that excess zinc is co-released with glutamate and enhances its neurotoxicity. Choi and co-workers reported that zinc caused apoptotic death of primary cultured cortical neurons[6]. They also revealed the accumulation of chelatable zinc in degenerating neurons

*To whom all correspondence should be addressed: Masahiro KAWAHARA, Ph.D., Department of Analytical Chemistry, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare, 1714－1 Yoshino-cho, Nobeoka-city, Miyazaki, 882－8508, Japan, Tel: (81) 982－23－5706, Fax: (81) 982－23－5708, E-mail: kawamasa@phoenix.ac.jp

of the hippocampus after transient global ischemia[7]. This zinc translocation occurred in vulnerable neurons in the hippocampus after transient global ischemia but before the onset of the delayed neuronal death, and enhanced the infarct[8]. These results firmly indicate zinc as a key factor in delayed neuronal death after the transient global ischemia which might be involved in the pathogenesis of vascular type of dementia[9]. Therefore, it is possible that substances which inhibit zinc-induced neurotoxicity will protect the delayed neuronal death after ischemia and could be a "seed" for the prevention or treatment of vascular type of dementia. In fact, administration of calcium EDTA (Ca-EDTA), a zinc-selective membrane-impermeable chelator, inhibited zinc-induced death of cultured cortical neurons[7], blocked the accumulation of zinc, protected the hippocampal neurons after transient global ischemia[10], and reduced the infarct volume[8].

Based on these findings, we have established a convenient and rapid screening system for protective substances of zinc-induced neurotoxicity and examined various agricultural products such as extracts of fishes, sea-foods, fruits, and vegetables[11]. Among tested, we found that the eel extract exhibited significant protective activity. We searched for active substances in the eel extract and revealed that carnosine (β-alanyl histidine), which is abundantly contained in the eel extract, protected GT1-7 cells against zinc-induced neuronal death.

Materials and Methods

Cell culture: GT1-7 cells were cultured as previously described[12]. Briefly, the cells were grown in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mixture F-12 Ham (F-12) supplemented with 10 % fetal calf serum. Upon reaching confluence, the cells were dissociated by incubation for 5 min at 37℃ in trypsin-EDTA solution. After enzymatic dissociation, the cells were resuspended in serum-free DMEM and plated on culture dishes. The cells were incubated in a humidified atmosphere of 93 % air and 7 % CO_2 at 37℃.

Cell viability assay: GT1-7 cells were plated on 96-well formatted culture dishes at the concentration of 5 x 10^5 cells/well. After 1 day *in vitro*, the solutions of $ZnCl_2$ (20-200 μM) were added to cell culture medium. After 24 h of the exposure, we examined viability of the cells using a commercially available kit (Cell Counting Kit, Dojindo Chemicals). The WST-1 assay used here is a modification of the MTT assay and measured the activity of the cellular mitochondrial dehydrogenase[13] using a microplate spectrophotometric reader (ImmunoMini NJ-2300, BioRad). The OD450-OD620 value represented the cell viability. To examine effects of fish extracts or carnosine (obtained from Sigma-Aldrich Ltd.), the solutions of various extracts were preadministered to culture media just prior to the exposure to zinc.

Morphological observation: GT1-7 cells were plated on polyethylenimine-coated coverslips at a concentration of 1.25 x 10^5 cells/cm^2 and maintained in serum-free DMEM for two days. After 24 h exposure to the solutions of $ZnCl_2$, the phase-contrast images of cells were observed under a microscope (IX71; Olympus).

Preparation of eel extracts: Eels were obtained from local commercial sources. The muscle of eels was added to the same volume of distilled water and homogenized. After centrifugation (20,000 g for 20 min), the supernatant was heated at 95℃ for 30 min, and centrifuged again. The supernatant was obtained by centrifugation at 20,000 g for 20 min, and then filtered through a 0.22 μm-pore filter and lyophilized. The final concentration of eel extracts was adjusted to 0.5 g fresh weight of eel muscle per mL with the appropriate amount of water.

Results

Development of screening system

We have already demonstrated that zinc caused marked death of GT1-7 cells (immortalized hypothalamic neu-

rons) in a dose-dependent and time-dependent manner[14-16]. GT1-7 cells were developed by genetically targeting tumorigenesis of mouse hypothalamic neurons and possess neuronal characteristics such as the extension of neuritis, the secretion of GnRH (gonadotropin-releasing hormone), and the expression of neuron-specific proteins or receptors[17]. Furthermore, we have revealed that GT1-7 cells are much more sensitive to zinc than are other neuronal cells[18, 19] including PC-12 cells, B-50 cells (neuroblastoma cell line), primary cultured neurons of rat cerebral cortex, and primary cultured neurons of rat hippocampus and exhibited the lowest viability after zinc exposure. Owing to these advantages, the GT1-7 cells provide a sensitive and convenient assay system for mass-screening of protective substances for zinc neurotoxicity. Using this system, we have revealed that sodium pyruvate significantly zinc neurotoxicity (Fig. 1).

Lee *et al.* have demonstrated that intracerebral administration of sodium pyruvate blocked the zinc accumulation as well as the degeneration of hippocampal neurons after ischemia[20]. Therefore, it is highly possible that this screening system is effective in exploring substances which inhibit neurodegeneration after ischemia.

Protective effects of the eel extract on zinc-induced neurotoxicity

Using this system, we examined inhibitory effects of various agricultural products or related compounds. Among tested, the water-soluble, heat-stable extract of eel significantly protected against GT1-7 cells zinc-induced neurotoxicity (Fig. 2). Zinc caused death of GT1-7 cells in a dose-dependent manner. The cell viability was decreased to 31.5 ± 3.1 % (mean ± S.E.M., n=6) compared to control after 24h exposure to 20 μM of zinc, Meanwhile, the cell viability of GT1-7 cells supplemented with 5 μL of the eel extract (corresponding to 2.5 mg of eel muscles) was 79.2 ± 8.3 % after zinc exposure.

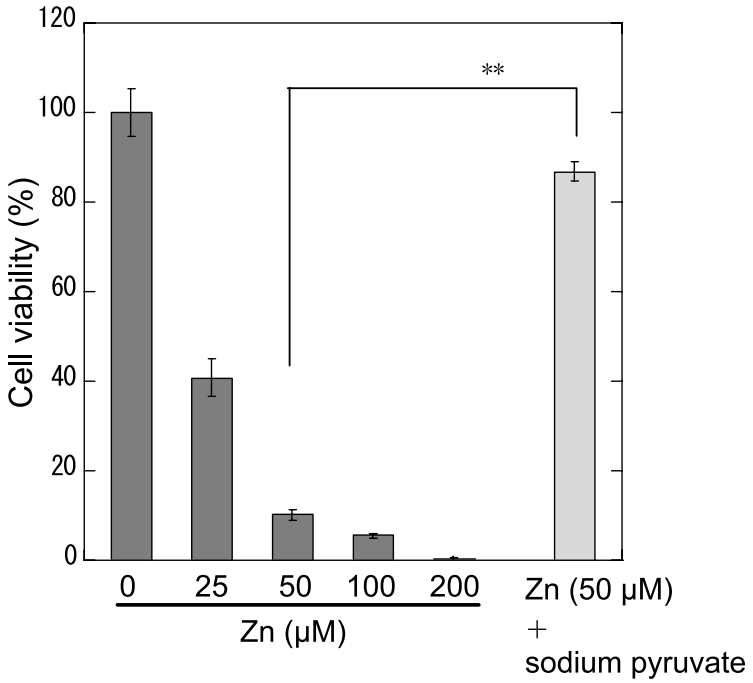

Fig. 1 Zinc-induced neurotoxicity on GT1-7 cells and protective effects of sodium pyruvate.
Solutions of $ZnCl_2$ (25-200 μM) was administered to GT1-7 cells at 1 day *in vitro*. After 24 h exposure, the cell viability was measured using WST-1 method. Sodium pyruvate (2 mM) was preadministered to GT1-7 cells prior to exposure to 50 μM of zinc. Data are means +/- S.E.M., n = 6; ** $p < 0.001$.

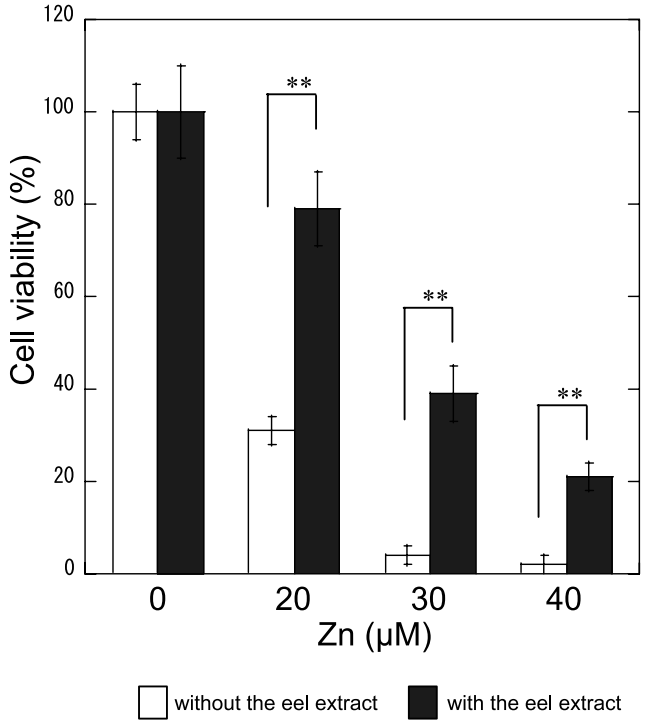

Fig. 2 Effects of the eel extract on zinc-induced neurotoxicity
The cell viability of GT1-7 cells after exposure to zinc (20-40 μM) with (black square) or without (open square) the eel extract (5 μL) was measured using WST-1 method. Data are means +/- S.E.M., n = 6; ** $p < 0.001$.

Figure 3 shows the phase-contrast images of GT1-7 cells after the experiment. GT1-7 cells exhibit sphere-like or neuronal-like shapes without exposure to zinc (control; Fig. 3(A)). However, exposure to zinc caused the cell shrinkages and morphologically abnormal apoptotic shapes (Fig. 3(B)). Supplementation with the eel extract recovered the morphological changes by zinc (Fig. 3(C)).

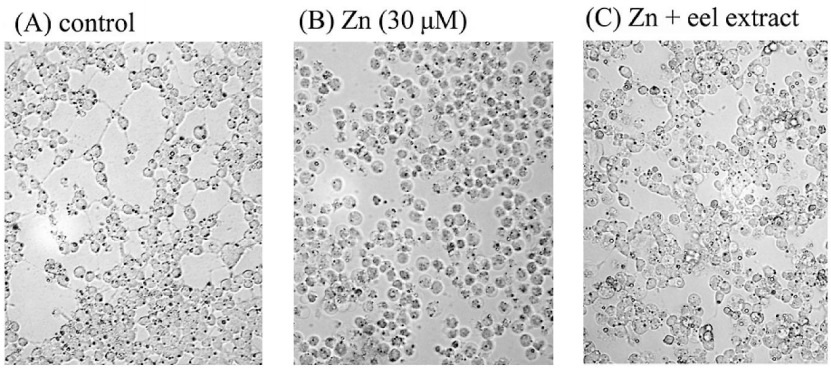

Fig. 3 Morphological changes of GT1-7 cells after exposure to zinc
GT1-7 cells were exposed to zinc (30 μM) with or without the the eel extract at 1 day *in vitro*. After 24 h, the morphological changes of GT1-7 cells were observed by phase-contrast microscopy. A: control, B: GT1-7 cells exposed to of zinc (30 μM), C: GT1-7 cells preadminiterated with 5 μL of the eel extract prior to Zn^{2+}. Bar represents 25 μm.

Protective effects of carnosine on zinc-induced neurotoxicity

We searched for active substances in the eel extract. Considering that the substances are water-soluble and heat-stable, we evaluated several hydrophilic substances known to exist in fishes and found that carnosine protected against GT1-7 cells from zinc-induced neurotoxicity in a dose-dependent manner (Fig. 4). Co-existence of 2 mM of carnosine blocked the toxicity induced by 30 μM of zinc and the cell viability was raised to 31.2 ± 2.4 %, while the viability of GT1-7 cells exposed to 30 μM zinc was 17.8 ± 1.1 % (mean ± SEM, n = 6).

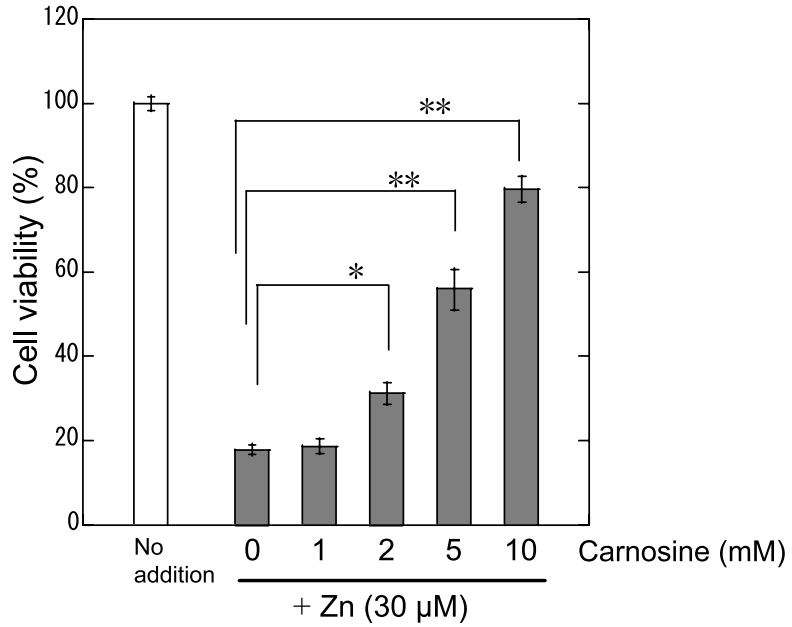

Fig. 4 Effects of carnosine on zinc neurotoxicity to GT1-7 cells
Various concentrations of carnosine were preadministered to GT1-7 cells prior to exposure to 30 μM of $ZnCl_2$. After 24 h, the cell viability was measured using the WST-1 method. Data are means +/- S.E.M., n = 6. $^{*}p < 0.01$, $^{**}p < 0.001$.

Using reversed-phase HPLC, the eel extract was revealed to contain much amount of carnosine. The concentration of carnosine in the eel extract was estimated to be approximately 1.2 mg/g of eel muscles (wet weight) (data not shown here). Thus, the final concentration of carnosine after administration of the eel extract was estimated to be approximately 2 mM in the experiment shown in Fig. 2. Mean while, analogues of carnosine such as anserine or alanyl histidine were not detected.

Discussion

Carnosine is a small dipeptide and is abundantly present in muscles of fishes, chickens, mammals[21]. Although its physiological roles remain elusive, it is suggested that carnosine plays important functions in pH balances in muscles after exercise[22]. It is also suggested that carnosine has the antioxidant activity and plays an endogenous suppressor of oxidative damages[23]. In the brain, carnosine also exists in the neurons olfactory bulb, and is secreted to synaptic clefts with excitatory neurotransmitter glutamate during the neuronal excitation[24]. The existence of carnosine in the glial cells was also reported[25]. Although the physiological roles of carnosine in the olfactory bulb are still unclear, it is hypothesized that carnosine might modulate neuronal functions. Considering that olfactory bulb neu-

rons are less sensitive to damages after ischemia compared to hippocampal neurons in spite of the accumulation of zinc, it is possible that carnosine plays protective roles in zinc-induced neurodegeneration after ischemia in the olfactory bulb. Furthermore, the content of carnosine is varied during development[26] and the content of carnosine in muscle is decreased in aged animals[27]. Therefore, the dietary supplementation of carnosine might be effective in the prevention or treatment for neurodegeneration after ischemia.

The molecular mechanism of carnosine for prevention of zinc-induced neuronal death is under investigation. Considering that carnosine has the ability of binding to zinc[28], the chelation of zinc may be based on the inhibitory effects. However, it is not plausible that from our preliminary experiments. The preincubation of zinc with carnosine did not enhance the inhibitory activity. Moreover, carnosine did not block the rise in the concentration of intracellular zinc (data not shown here). Taurine has the anti-oxidant activity similar to carnosine, however, it has no protective activity for zinc-induced neurotoxicity. Thus, it is possible that other factors may underlie the inhibitory effects of carnosine in zinc-induced neuronal death. Further research about the mechanism of zinc-induced neurotoxicity will lead to the clue of drugs for neurodegeneration after ischemia, and finally to vascular type of dementia.

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