Trace Nutrients Research 25 : 41−45 (2008)

Original Article

Improvement of Screening for Protective Substances against Zinc-induced Neuronal Cell Death

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Summary

The inter-neuronal movement of zinc ions is a key mechanism involved in ischemic neuronal death. Therefore, zinc-induced neuronal cell death is a suitable phenomenon to observe for examining the neurodegenerative damage following ischemia. We have established a convenient and rapid screening system for protective substances against zinc-induced neuronal cell death and isolated carnosine, pyruvate, α-tocopherol, and gadolinium as protective substances. In this study, we modified the screening system to particularly identify substances with weak protective activity. The modifications, earlier administration of the samples and zinc, and the measurement of cell mortality, allowed the present assay to perform the sensitive and reliable detection of protective activity against zinc-induced GT1-7 cell death. Dose-dependency of pyruvate showed that the present assay was improved specially in detecting weak protective activity compared to the previous one. Using the present assay, numerous protective activities could be significantly distinguished from the real samples. The screening method with the present assay will extend the possibilities of screening samples.

Introduction

Zinc−an essential trace element in the body−is concentrated in the central nervous system and is released during synaptic activity or membrane depolarization[1−3]. Particularly following brain injury such as global ischemia, presynaptic zinc is specifically accumulated in the degenerating neurons of the hippocampal CA1 as well as other vulnerable neurons in the cortex, amygdala, striatum, and thalamus, thus resulting in neurodegeneration[4,5]. An experiment using a metal chelator revealed that the inhibition of such Zn^{2+} accumulation drastically reduced ischemic neuronal cell death, strongly suggesting that the interneuronal movement of Zn^{2+} is a key mechanism involved in ischemic neuronal death[5]. These results suggested that zinc-induced neuronal cell death is a suitable phenomenon to observe for examining the neurodegenerative damage following brain injury such as transient ischemia.

We have established a convenient and rapid screening system for protective substances against zinc-induced neurodegeneration[6]. The system utilizes GT1-7 cell, which is derived from the immortalized hypothalamic neurons[7−9] and is extremely sensitive to zinc. GT1-7 cells are not affected by the treatment of glutamate [10], which has been reported to be the main inducer of cell death following transient ischemia. Therefore, the use of GT1-7 cells makes our screening system sensitive and specific to zinc-induced neuronal cell death. In actuality, by using this screening system, we have isolated protective substances such as carnosine, pyruvate, α-tocopherol, and gadolinium[6,10−12]. These protective substances may prove to be strong candidates for the development of a medicine for reducing neurodegenerative damage following transient ischemia[13] ; this is because zinc contributes to brain injury after ischemia. In this study, we improved our screening system for the purpose of further isolating protective substances, especially substances with weak protective activity. This system should to be utilized for isolating various screening samples that contained small amount of the protective substance.

Materials and Methods

1. Materials

Caspase-3 inhibitor, Z-DEVD-FMK was obtained from R&D systems (USA), and Calpain inhibitor, Mu-Val-HPh-FMK from MP Biomedicals (USA). They were dissolved in dimethylsulfoxide at a final concentration of 20 mM. The screening samples from the bioactive materials were pre-

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pared in water. The details are confidential due to patent submission.

2. Cell culture and viability assay

GT1-7 cell were grown in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham (DMEM/F-12) supplemented with 10 % fetal bovine serum. The culture was maintained in a humidified incubator at 37 ℃ and 7 % CO_2. After enzymatic digestion using trypsin, the GT1-7 cells were resuspended in serum-free DMEM/F-12 and plated on 96-well formatted culture dishes at a concentration of 1×10^6 cells/well. Zinc and/or tested samples were administrated to the plating cells at 6 or 24 h after dividing the cells. The cell culture medium was replaced with a fresh medium after a 20-h exposure to zinc, and then incubated for 1 h at 37℃. The aliquots of the replaced medium were transferred to a new 96-well plate, and the lactate dehydrogenase activity released from the cells was determined by a colorimetric cytotoxicity assay LK 100 kit (Oxford Biomedical Research, UK) with measuring OD_{490} for determining the rate of cell death. In addition, the activity of the mitochondorial dehydrogenase in the cell was determined using a WST-1 assay cell counting kit (Dojindo Chemicals, Japan) with measuring $OD_{450}-OD_{620}$ value for determining cell viability. The absorbance was measured by using a microplate spectrophotometric reader.

3. DNA laddering assay

GT1-7 cells (8×10^6 cells), which had been treated with or without 30 μM $ZnCl_2$ for 24 h, were suspended in an appropriate lysis buffer containing 0.5 % triton X-100 at 4℃ for 10 min, and sequentially treated with RNase A at 37℃ for 1 h and proteinase K at 50℃ for 1 h. The redundant substances such as protein were removed by phenol-chloroform extraction, and the DNA was concentrated by a precipitation with isopropanol. The samples were subjected to electrophoresis with 2 % agarose gel, and were stained with ethidium bromide.

4. Statistical analysis

For a statistical analysis, the Bonferroni test was employed to compare the zinc-treated cells with the protected cells. The data are expressed as means ± SD. $P < 0.01$ was considered as a significant value.

Results and Discussion

1. Early administration of samples and zinc allowed a sensitive assay for protective activity against zinc-induced neuronal cell death.

We have shown that GT1-7 cells undergo apoptotic cell death after zinc treatment by using a terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling[7] (TUNEL), and also showed the DNA laddering phenomenon in zinc-treated GT1-7 cells (Fig. 1), which is a typical marker of the apoptosis event. Both caspase-3 and calpain, which are classified as cystein protease, have been reported to be key mediators of apoptosis ; thus, we further tested the specific inhibitors of caspase-3 and calpain for protecting zinc-induced GT1-7 cell death (Fig. 2). Although both of the inhibitors (40 μM at final concentration) showed a tendency to recover cell viability, the significant difference between the viabilities of treated and non-treated cells was difficult to observe in the standard assay procedure (Fig. 2a, b). In order to identify such weak protective activity, we altered the various assay conditions and examined these inhibitors. In consequence, we found that early treatment of the inhibitors and zinc yielded good results. Both inhibitors and zinc were administrated to the cells at 6 h after dividing the cells on the 96-well plates, whereas both were administrated at 24 h after dividing the cells in the standard assay. Using the improved assay, both inhibitors of caspase-3 and calpain significantly recovered the viabilities of zinc-treated cells (Fig. 2c, d). To the extent of our knowledge, this is the first report to

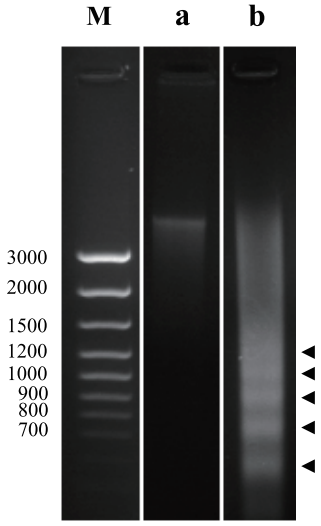

Fig. 1 Electrophoresis of DNA from GT1-7 cells treated with (lane b) or without (lane a) zinc (30 μM in final concentration). The DNA laddering phenomenon is indicated by arrowheads on the right-hand side of the photograph. The marker DNA ladder is separated in lane M and the lengths in bp are shown on the left-hand side of the photograph.

show the contribution of calpain to the apoptotic pathway of the GT1-7 cells.

However, the precise assay seemed to be difficult because the cell viability of zinc-treated cells in the improved assay appeared to be at a higher level than that in the standard assay, although the phenomenon is reproducible and probably results from the freshness of the cells. Therefore, we altered the assay procedures to measure the cell mortality, i.e., the activity of released lactate dehydrogenase (Fig. 2e and f). Cell mortality is a more reliable index for the evaluation of weak protective activity than cell viability under this condition (compare c with e, and d with f). As a result, two modifications, the early administration of samples and zinc and the measurement of cell mortality, are included in the improved assay for weak protective activity against zinc-induced neuronal cell death.

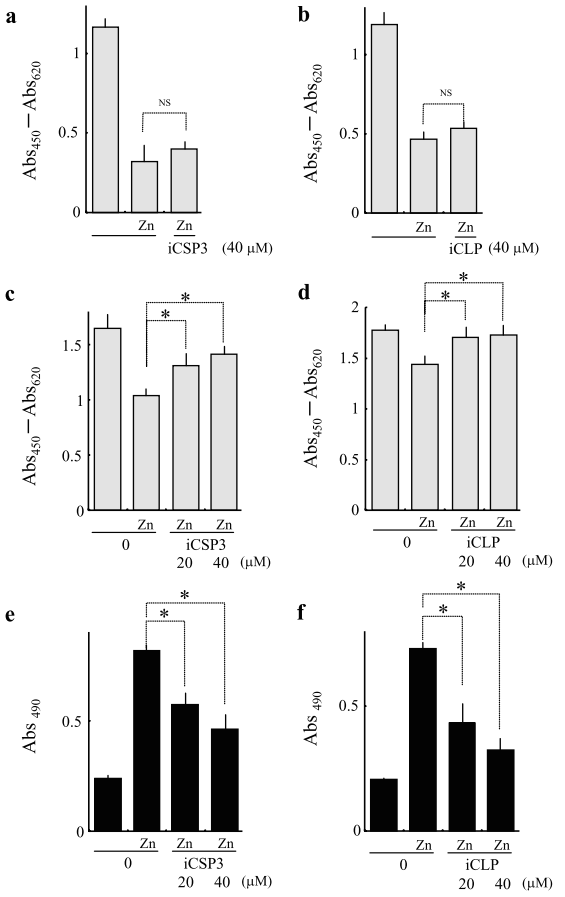

Fig. 2 Effect of the timing of zinc treatment on detection sensitivity for protection activity against zinc-induced cell death. Zinc (35 μM in final concentration) was administrated to GT1-7 cells at 6 h (c–f) or 24 h (a, b) after dividing the cells from the flask. Cell permeable inhibitor for caspase-3 (iCSP3 ; a, c, e) or for calpain (iCLP ; b, d, f) was treated on the cell just before zinc administration. The viabilities (a–d) or mortality (e, f) of the treated cells was determined by WST-1 assay or cytotoxicity assay 20 h after zinc administration. The treated conditions (zinc administration and inhibitor concentrations) are indicated under each figure. The data are expressed as mean ± SD, n = 6. * indicates the respective significances at $P < 0.01$ vs. the cells treated with $ZnCl_2$ alone.

2. Detection sensitivity for protective activity of pyruvate of the improved assay was higher than that of the standard.

To compare the sensitivity for detecting the protective activity against zinc-induced neuronal cell death, the dose-dependencies of pyruvate on the protective activity were determined in both the standard and improved assays. Pyruvate has been reported to prevent zinc-induced neuronal cell death through a mechanism of recovering the energy deficiency that is attributable to the loss of NAD^+ and inhibition of glycolysis[14] ; in other words, pyruvate prevents the zinc-induced necrosis pathway of the cell. In the

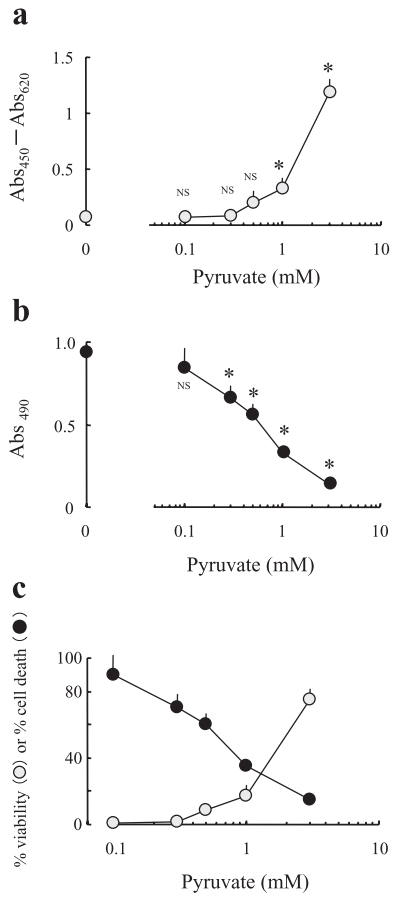

Fig. 3 Comparison of dose-dependencies of pyruvate for protective activity between the improved and standard assays. Zinc (35 μM in final concentration) was administrated to GT1-7 cells at 6 h (b) or 24 h (a) after dividing the cells from the flask. Pyruvate, the final concentrations of which are indicated under each figure, was treated to the cell just before zinc administration. The viabilities (a) or mortality (b) of the treated cells was determined by WST-1 assay or cytotoxicity assay 20 h after zinc administration, respectively. Both relative activities are plotted on figure (c), in which the viability of the non-treated cells or the mortality of the zinc-treated cells was assumed to be 100 %. The data are expressed as mean ± SD, n = 6. * indicates the respective significances at $P < 0.01$ vs. the cells treated with $ZnCl_2$ alone.

standard assay, pyruvate significantly prevented the zinc-induced GT1-7 cell death at the concentration of more than 1 mM (Fig. 3a), and 3 mM pyruvate recovered the cell viability up to approximately 80 %, compared to the viability of the non-treated cells (c). On the other hand, in the improved assay, it significantly prevented cell death at the concentration of more than 0.3 mM (Fig. 3b), and 3 mM pyruvate recovered up to more than 80 % (c). Figure 3c clearly shows that the improved assay advances particularly in detecting the weak activity for protecting against zinc-induced neuronal cell death. Taken together, these results indicate that the improved assay is sensitive in the protection activity both for apoptosis and necrosis cell deaths.

3. The improved assay clearly distinguished the protective activity of real samples compared to the standard assay.

We examined the screening ability of the improved assay using real samples, compared to that of the standard assay (Fig. 4). Ten samples, A-J, were extracted from some bioactive materials and dissolved in water. The protective activities were compared between the improved assay with measuring the cell mortality (Fig. 4a) and the standard assay with measuring the cell viabilities (b). The improved assay clearly showed that seven of ten samples significantly recovered the zinc-induced neuronal cell death, while the standard assay showed the recoveries of only three samples. In addition, comparing Figure 4a with Figure 4b, the improved assay provided a precise and reliable screening style for the samples with weak protective activity in the event of zinc-induced neuronal cell death.

In conclusion, we established a highly sensitive screening system for protective substances against zinc-induced neuronal cell death. Since the protective substances may prove to be strong candidates for the development of medicine for reducing neurodegenerative damage following transient ischemia, the improved sensitive assay extend the screening targets to various samples including those with weak activity. Using the improved assay, we will further investigate the apoptotic mechanisms in the GT1-7 cells.

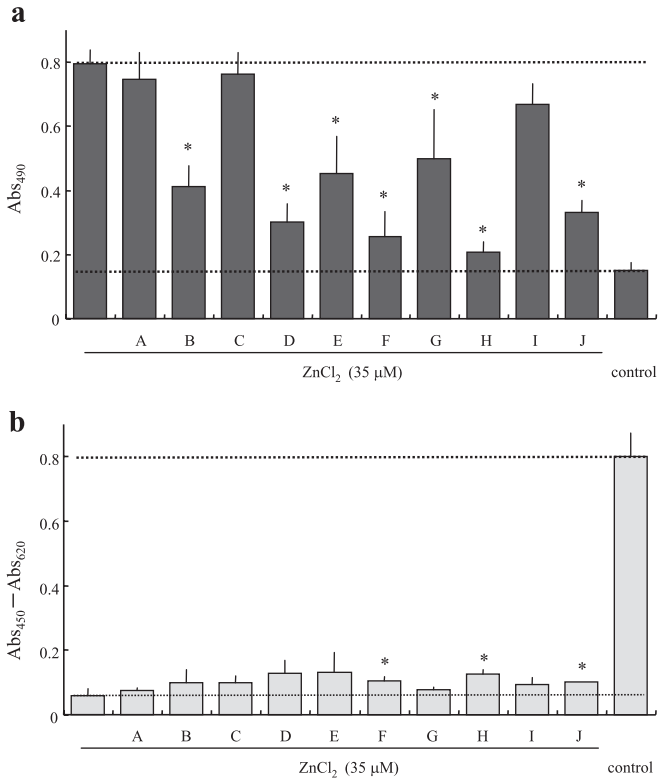

Fig. 4 Screening of the real sample in the improved assay (a) and the standard assay (b). Zinc (35 μM in final concentration) was administrated to GT1-7 cells at 6 h (a) or 24 h (b) after dividing the cells from the flask. The practical screening samples (A−J), obtained from some bioactive materials, was treated to the cell just before zinc administration. The viabilities (b) or mortality (a) of the treated cells was determined by WST-1 assay or cytotoxicity assay 20 h after zinc administration. The treated conditions are indicated under each figure. The data are expressed as mean ± SD, n = 6. * indicates the respective significances at $P < 0.01$ vs. the cells treated with $ZnCl_2$ alone.

Acknowledgments

Part of this work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and a grant from the Ministry of Economy, Trade and Industry (Kyushu bureau in Japan).

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