Glutathione Depletion Promotes Aluminum-Mediated Cell Death of PC12 Cells

Eiko SATOH, Morihiro OKADA, Tsuneo TAKADERA, and Takao OHYASHIKI*

Department of Clinical Chemistry, Faculty of Pharmaceutical Sciences, Hokuriku University; Kanagawa-machi, Kanazawa, Ishikawa 920–1181, Japan. Received November 16, 2004; accepted February 28, 2005

Exposure of rat phenochromocytoma cells (PC12 cells) to aluminum maltolate complex, Al(maltol)$_3$, induced a decrease in intracellular glutathione (GSH) concentration, resulting in a facilitated release of lactate dehydrogenase (LDH) from the cell and an increase in trypan blue-stained cells. Similar phenomena were observed as the cells were treated with L-buthione-[*S,R*]-sulfoximine (BSO) in the presence of Al(maltol)$_3$. On the other hand, treatment of PC 12 cells with BSO alone in the absence of Al(maltol)$_3$ did not affect the cell viability. Pre-treatment of PC12 cells with *N*-acetylcysteine (NAC) for 30 min before a 48 h-exposure to Al(maltol)$_3$ effectively protected the cells from Al(maltol)$_3$ toxicity by increasing intracellular GSH concentration. NAC also effectively inhibited reactive oxygen species (ROS) generation induced by treatment of the cells with Al(maltol)$_3$. However, several lipophilic radical scavengers such as α-tocopherol and 3(2)-*tert*-butyl-4-hydroxyanisole, and an iron chelator, desferrioxamine, did not prevent Al(maltol)$_3$-mediated ROS production or the decrease of cell viability. Based on these results, we discussed the role of intracellular GSH against the onset of aluminum toxicity in the context of ROS production.

Key words aluminum; cell death; glutathione; oxidative stress; reactive oxygen species

Aluminum (Al) has been implicated as an etiologic factor in neurological disorders including Alzheimer's disease[1,2] and Parkinson's dementia syndrome.[3,4] In fact, it was reported to accumulate in high concentrations within the neurofibrillary tangle bearing neurons of patients with Alzheimer's disease.[1]

An increasing amount of evidence reported in model studies using cultured cells and animals has confirmed the fact that Al can have a severe neurotoxic effect. Suarez-Fernandez *et al*. have reported that prolonged exposure of cultured astrocytes and neurons to aluminum chloride results in a decrease in cell viability.[5] We have also recently reported that treatment of PC12 cells with aluminum maltolate complex induces cell death *via* apoptosis, and nerve growth factor effectively prevents this cell death.[6] In addition, Savory *et al.* have demonstrated that the administration of aluminum maltolate complex to rabbit results in marked neurofibrillary degeneration.[7] These findings strongly suggest that Al ions are responsible for the development of neurodegenerative disorders, although the cellular and molecular mechanism by which Al exerts its neurotoxic effects remains unclear.

Recently, several investigators have proposed the involvement of ROS generation in the onset of Al neurotoxicity.[6,8] In addition, there are several studies which show that Al^{3+} promotes iron-dependent lipid peroxidation in model membranes, such as phospholipid liposomes and membranes.[9—14] We have also recently reported that the stimulatory effect of Al^{3+} on iron-dependent lipid peroxidation in phosphatidylcholine liposomes is further enhanced under acidic conditions.[15] These results suggest that oxidative stress and/or membrane lipid peroxidation linked with ROS generation in cells may be an important mechanism for Al toxicity development.

It is well known that reduced glutathione (GSH) is the most abundant free cellular anitoxidant *in vivo*.[16] In addition, it has been reported that GSH metabolic pathways contribute to protection against oxidative events in the brain, because GSH effectively reduces peroxides in a non-enzymatic reaction.[17] Immunocytochemical studies show that GSH is present in high concentrations in both astrocytes and neurons.[18—20] Campbell *et al.* have recently reported that Al produces a marked facilitation of ROS production and a marked decrease in GSH concentration in rat glioma cells.[8] In addition, it has been reported that the lowering of intracellular GSH levels is involved in the onset of neuronal cell death.[21,22] These findings propose the importance of GSH in the survival mechanism in neuronal cells.

In the present study, we demonstrated that depletion of intracellular GSH is a trigger of the onset of Al cytotoxicity *via* ROS production.

MATERIALS AND METHODS

Materials 3-Hydroxy-2-methyl-4-pyron (maltol), *N*-acetylcysteine (NAC), glutathione (reduced type, GSH), α-tocopherol, 3(2)-*tert*-butyl-4-hydroxyanisole (BHA), glutathione (reduced form, GSH), glutathione reductase (GR) and glutathione *S*-transferase (GST) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Monochlorobimane (mBCl) was from Funakoshi (Kumamoto, Japan). A stock solution (40 mM) of mBCl was prepared by dissolving it in acetonitrile, followed by storage at −20 °C in the dark. L-Buthionine-[*S,R*]-sulfoximine (BSO) and desferrioxamine (DFO) were obtained from Sigma (St. Louise, MO, U.S.A.). 6-Carboxy-2′,7′-dichloro-dihydrofluorescein diacetate, di(acetoxymethyl ester) (C-DCDHF-DA) was from Molecular Probes, Inc. (Eugene, OR, U.S.A.). All chemicals used were of the purest grade commercially obtainable.

Preparation of Aluminum Maltolate Complex Al(maltol)$_3$ was prepared according to the procedure described by Finnegan *et al.*[23] A stock solution (50 mM) of Al(maltol)$_3$ was prepared in phosphate-buffered saline (pH 7.4) and sterilized using a 0.22 μM filter.

Cell Culture PC12 cells were cultured in 35 mm dishes coated with poly-D-lysine at a density of approximately 3.5×10^5 cells per dish in Dulbecco's modified Eagle medium

* To whom correspondence should be addressed. e-mail: t-ohyashiki@hokuriku-u.ac.jp

(DMEM) supplemented with 5% precolostrum newborn calf serum and 5% horse serum at 37 °C under 95% air/5% CO_2. They were allowed to develop for 24 h before exposure to $Al(maltol)_3$, unless otherwise specified. Control cells were cultured in the presence of three times the quantity of maltolate in the place of $Al(maltol)_3$ employed. Morphological changes in cells were checked throughout the course of the experiment by phase-contrast microscopy (Olympus IX 70-S8F microscope).

Cell Viability Cell viability was assessed by lactate dehydrogenase (LDH) release measurement and the trypan blue exclusion method. (a) LDH release measurement: The reaction was started by the addition of an aliquot (250 μl) of cultured medium to a medium (750 μl) containing NADH (88 μg/ml), pyruvate (100 μM) and 100 mM phosphate buffer (pH 7.5). The rate of change in the absorbance of NADH at 340 nm (ΔA_{340}) was measured within 1 min using a Hitachi spectrophotometer 100-60. (b) Trypan blue exclusion: The cells were stained by 0.4% trypan blue solution for 5 min at 37 °C under 95% air/5% CO_2.

Glutathione Determination The GSH concentrations were assayed by the procedure reported by Schrieve *et al.*[24] The cells were incubated with 20 μM mBCl (as a final concentration) for 10 min at 37 °C under 95% air/5% CO_2. Then, the treated cells were washed at once with Ca^{2+}/Mg^{2+}-free phosphate buffered saline (CMF-PBS) and scraped with a rubber stick using 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA. After sonication for 1 min with a Ultrasonic Homogenizer UH-150, the cell suspension was centrifuged at 1500×***g*** for 5 min. The fluorescence intensity of the supernatant was measured with excitation and emission wavelengths at 395 nm and 475 nm, respectively, using a Hitachi spectrofluorometer F-4500 (Hitachi, Ltd., Japan). The standard curve was made as follows. Several concentrations of GSH (0—5 nmol) were pre-incubated with NADPH (0.2 mg/ml), GR (0.6 units/ml) and 1 mM EDTA in 100 mM phosphate buffer (pH 7.4) for 3 min at 37 °C. After incubation with GST (0.1 units/ml) and 20 μM mBCl for 10 min at 37 °C, the fluorescence intensity was measured.

Assay of ROS Generation ROS generation was measured using a fluorescence dye, C-DCDHF-DA, as described in our previous paper.[6] The pre-loaded cells with 5 μM C-DCDHF-DA (as a final concentration) for 60 min at 37 °C were incubated with 150 μM $Al(maltol)_3$ or 450 μM maltolate alone for 24 h, unless otherwise specified. Then, the cells were washed twice with CMF-PBS and solubilized with 2% SDS. After centrifugation for 20 min at 1500×***g*** at room temperature, the fluorescence of the supernatant was measured with the excitation and emission wavelengths at 480 and 523 nm, respectively. The fluorescence intensity (arbitrary unit) was expressed as the value per μg protein.

Protein Determination Protein concentration was determined by the procedure described by Lowry *et al.* using bovine serum albumin as the standard.[25]

Statistical Analysis Data are presented as the mean± S.D. values of three different experiments. The data were analyzed by an ANOVA Bonferroni's multiple *t*-test.

RESULTS

Effects of $Al(maltol)_3$ Treatment on Cell Viability and GSH Content The effect of $Al(maltol)_3$ on the cell viability of PC12 cells was examined in relation to intracellular GSH concentration.

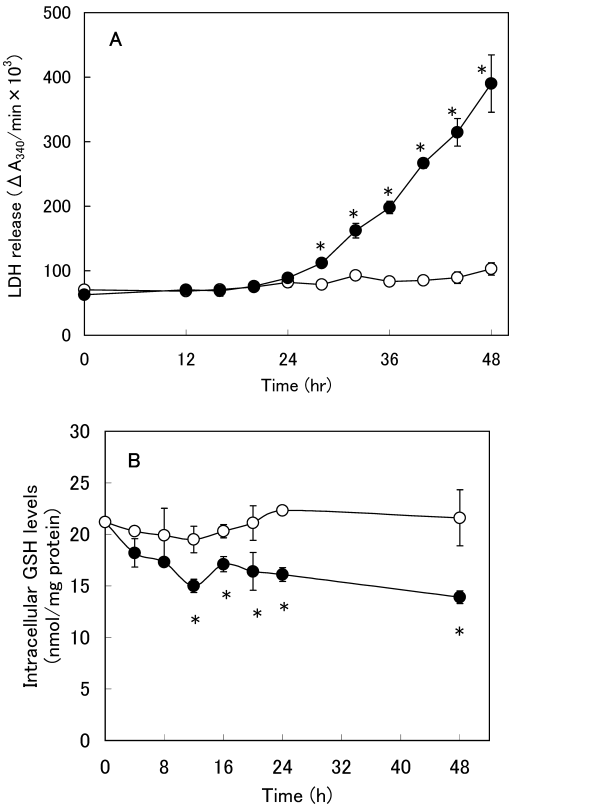

Fig. 1. Time Course of $Al(maltol)_3$-Induced LDH Release and GSH Depletion

(A) LDH release, (B) GSH concentration. The symbols ○ and ● represent control (450 μM maltolate) and 150 μM $Al(maltol)_3$-treated cells, respectively. $*p<0.01$ *vs.* control cells in each time period.

Figure. 1A shows the typical result of LDH release induced by treatment of the cells with 150 μM $Al(maltol)_3$. When the cells were exposed to 150 μM $Al(maltol)_3$, an appreciable release of LDH from the cells was initiated after the lag period of 28 h. On the other hand, the rate of LDH release in the absence of $Al(maltol)_3$ ($\Delta A_{340}=70.3\pm1.2\times10^{-3}$/min) was quite slow until a 48 h-exposure ($\Delta A_{340}=89.5\pm2.5\times10^{-3}$/min). In contrast, the intracellular GSH concentration decreased in a time-dependent manner, from 21.2± 0.4 nmol/mg protein to 13.9±0.6 nmol/mg protein after a 48-h exposure to $Al(maltol)_3$ (Fig. 1B). However, the GSH concentration of the control cells with 450 μM maltolate did also not change after a 48 h-exposure (21.6±2.7 nmol/mg protein).

The cytotoxicity of $Al(maltol)_3$ against PC12 cells was also confirmed by an increase in trypan blue-stained cells (Fig. 2). In this case, again, maltolate alone (450 μM) also did not induce an increase of trypan blue-stained cells during a 48 h-exposure.

Effects of GSH Synthetase Inhibitor To determine whether the intracellular GSH level is directly involved in an $Al(maltol)_3$-induced decrease in cell viability, PC12 cells were pre-treated with various concentrations of BSO, a selective inhibitor of glutamylcysteine synthetase.[26]

When the cells were incubated with BSO, the intracellular GSH concentration decreased depending on the concentration of the inhibitor, and almost reached a constant level

above 50 μM GSH (Fig. 3).

Table 1 shows the effects of BSO treatment on LDH release from the cells in the absence and presence of Al(maltol)$_3$. In this experiment, PC12 cells were pre-treated with various concentrations (1—50 μM) of BSO before a 24-h exposure against 150 μM Al(maltol)$_3$. As shown in the table, LDH release in the absence of Al(maltol)$_3$ was not affected by the decreasing concentration of intracellular GSH. On the other hand, LDH release from the cells pre-treated with BSO was facilitated by exposure to 150 μM Al(maltol)$_3$, depending on the BSO concentration employed. The effects of BSO-pre-treatment on the cell morphology in the absence and presence of Al(maltol)$_3$ were assessed by observation of the cultures under phase-contrast optics.

As shown in Fig. 4, a 24 h-exposure of the cells without BSO-treatment for 150 μM Al(maltol)$_3$ did not show any appreciable changes in morphology. However, treatment of BSO-pre-treated cells with Al(maltol)$_3$ at the same concentration for 24 h caused a marked shrinkage of the cell body and an aggregation of the cells. These morphological changes in the cells coincide with those of the 48-h exposed

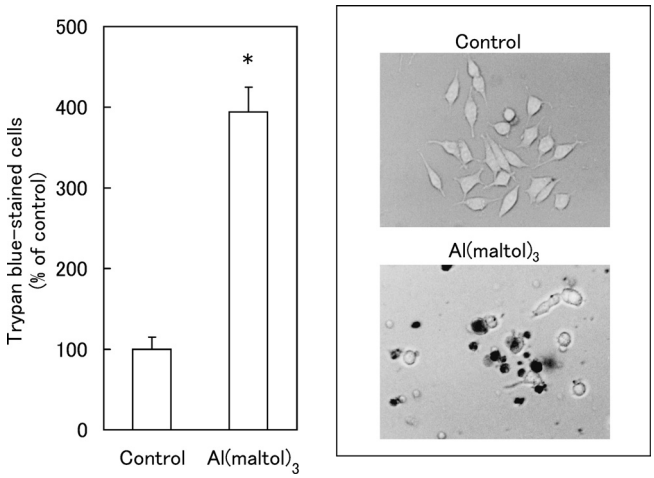

Fig. 2. Trypan Blue-Stained Cells after 48 h Exposure to Al(maltol)$_3$

The concentrations of maltolate (control) and Al(maltol)$_3$ were 450 and 150 μM, respectively. The number of dye-stained cells expressed is relative to that of the control cells.

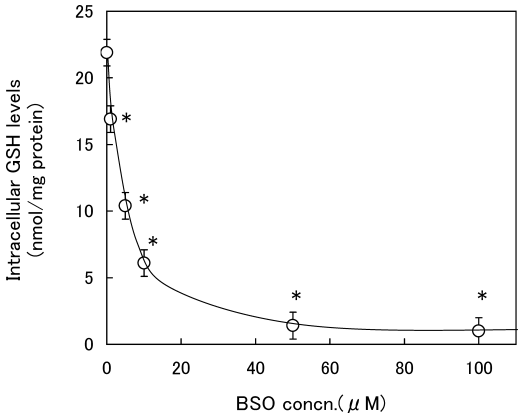

Fig. 3. Effects of Increasing Concentrations of BSO on Intracellular GSH Concentration

The cells were pre-treated with various concentrations (1 to 100 μM) of BSO for 24 h before 24 h exposure to 150 μM Al(maltol)$_3$. $*p<0.01$ *vs*. control cells without BSO.

Table 1. Effects of Increasing Concentrations of BSO on Al(maltol)$_3$-Induced LDH Release

BSO concn. (μM)	LDH release (ΔA_{340}/min$\times 10^3$)	
	Control	Al(maltol)$_3$-treated
0	77.5±1.9	77.0±5.9
1	77.9±1.3	82.9±6.3
5	75.6±3.9	93.1±6.2*
50	76.8±4.2	127.5±6.3*

The experimental conditions are the same as those described in the legend to Fig. 1, except the exposure period against Al(maltol)$_3$ is 24 h. $*p<0.01$ *vs*. control cells in each system.

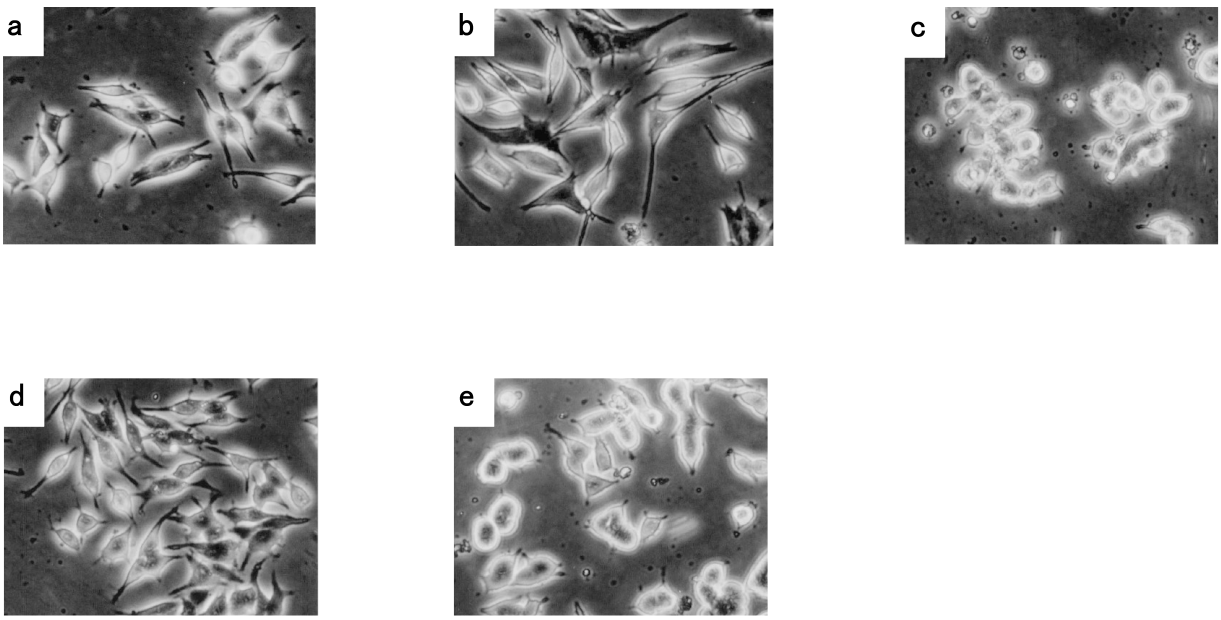

Fig. 4. Morphological Changes in BSO-Pre-Treated Cells Induced by Al(maltol)$_3$ Treatment

The cells were pre-treated with 50 μM BSO for 24 h before Al(maltol)$_3$ treatment, and then exposed to 150 μM Al(maltol)$_3$ for 24 h. a, control cells (450 μM maltolate); b, Al(maltol)$_3$-treated cells for 24 h; c, Al(maltol)$_3$-treated cells for 48 h; d, control cells with BSO for 24 h; e, Al(maltol)$_3$-treated cells with BSO.

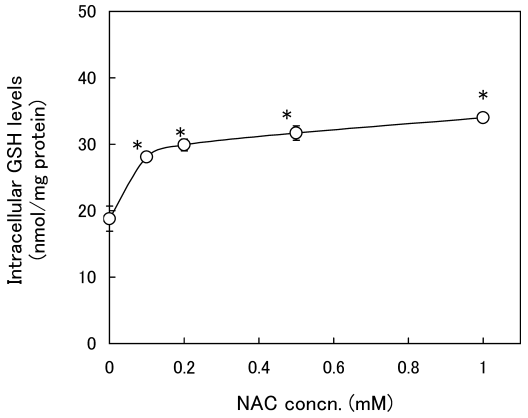

Fig. 5. Effects of NAC Treatment on Intracellular GSH Concentration

The cells were pre-treated by NAC before 48 h exposure to Al(maltol)$_3$. Other experimental conditions are the same as those described in the legend of Fig. 2. $*p<0.01$ *vs.* control cells in each system.

Table 2. Protection Effects of NAC against Al(maltol)$_3$-Induced Cell Injury

System	NAC	LDH release (ΔA_{340}/min$\times 10^3$)	Trypan blue-stained cells (% of control)
Control	−	70±2	100±15
Al(maltol)$_3$-treated	−	258±7	394±31
	+	150±10*	192±34*

The cells were pre-treated with NAC before a 48 h exposure to 150 μM Al(maltol)$_3$. Other experimental conditions are the same as those described in the legend of Fig. 2 and Table 1. $*p<0.01$ *vs.* Al(maltol)$_3$-treated cells without NAC in each system.

cells against 150 μM Al(maltol)$_3$. From these results, it is clear that BSO treatment of the cells facilitates Al(maltol)$_3$-induced changes in the cell morphology.

Effect of *N*-Acetylcysteine Next, we examined the effects of increasing concentrations of NAC, a precursor of GSH biosynthesis,[27] on the Al(maltol)$_3$-induced decrease in cell viability to further confirm the role of intracellular GSH in the onset of Al(maltol)$_3$ toxicity.

As shown in Fig. 5, pre-treatment of PC12 cells with 1 mM NAC for 30 min before Al(maltol)$_3$ treatment caused an increase in intracellular GSH levels, depending on the concentration of NAC. Table 2 shows the effects of NAC treatment on cell viability in the presence of 150 μM Al(maltol)$_3$. It is clear that NAC effectively prevented Al(maltol)$_3$-induced facilitation of LDH release and an increase of trypan blue-stained cells. The protection of Al(maltol)$_3$-induced facilitation of LDH release by NAC treatment was also observed in BSO (50 μM, 30 min)-treated cells (data not shown).

Effects of NAC and other antioxidants on ROS Production The effects of several antioxidants and/or radical scavengers, as well as iron chelators, on cellular ROS production were examined using a fluorescence dye C-DCDHF-DA.

As can be seen in Fig. 6A, 24 h-exposure of PC12 cells to 150 μM Al(maltol)$_3$ resulted in a marked increase of the fluorescence intensity. On the other hand, pre-treatment of the cells with 1 mM NAC for 30 min before Al(maltol)$_3$ treatment effectively prevented the fluorescence development. In contrast, pre-treatment of the cells with lipophilic radical scavengers such α-tocopherol and BHA, and with an iron chelator DFO, did not prevent Al(maltol)$_3$-induced fluorescence development. The LDH release induced by Al(maltol)$_3$ treatment was also not inhibited by the addition of these lipophilic compounds (Fig. 6B).

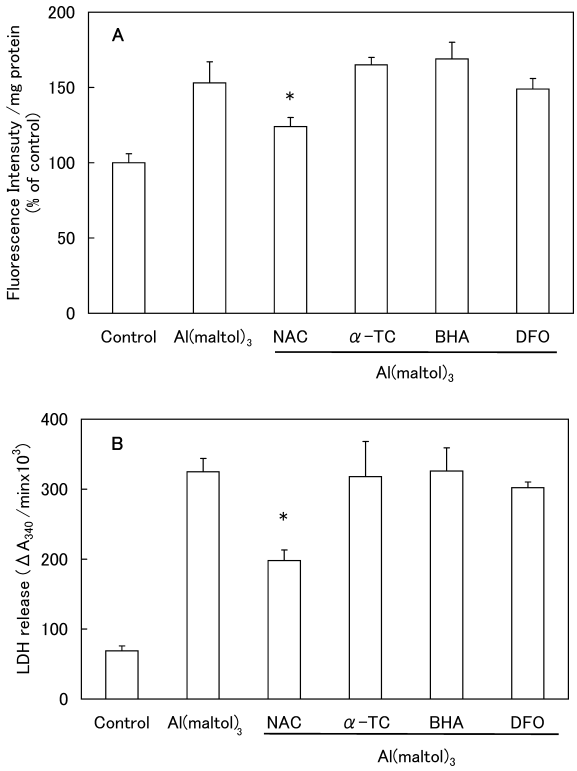

Fig. 6. Effects of NAC and Other Antioxidants on ROS Production

The fluorescence-loaded cells were pretreated with NAC (1 mM), α-tocopherol (50 μM), BHA (50 μM) or DFO (50 μM) for 30 min and then exposed to 150 μM Al(maltol)$_3$. The fluorescence intensity (FI) and LDH release were measured after 24 and 48 h, respectively. (A) ROS production. The FI expressed are relative to that of control cells without antioxidants. (B) LDH release. $*p<0.01$ *vs.* control cells in each system.

DISCUSSION

There is an increasing amount of evidence which suggests the involvement of the Al ion in a variety of neurogenerative disorders such as Alzheimer's disease.[1,2] In the previous paper, we reported that the accumulation of Al in PC12 cells induces a decrease in cell viability through apoptotic cell death, depending on the intracellular Al levels.[6]

In the present study, we demonstrated that intracellular GSH plays an important role in the onset mechanism of Al toxicity for PC12 cells. Exposure of PC12 cells to Al(maltol)$_3$ resulted in a facilitation of LDH release from the cells (Fig. 1A), as well as an increase in trypan blue-stained cells (Fig. 2) with the decrease in intracellular GSH concentration (Fig. 1B). Similar phenomena were also observed by pre-treatment of the cells with BSO before a 24 h-exposure to Al(maltol)$_3$ (Fig. 3, Table 1). However, treatment of the cells with BSO alone in the absence of Al(maltol)$_3$ did not induce an appreciable release of LDH from the cells, regardless of the loss of intracellular GSH, suggesting that depletion of cellular GSH is not a direct trigger of a decrease in cell viability. Similar observations have also been made in human monocytic cells[28] and human neurotrophils.[29] From these results, therefore, it is strongly suggested that the lowering of intracellular GSH concentration is not directly involved in cell death, but it promotes the onset of Al-

$(maltol)_3$-induced cell death of PC12 cells.

The results of fluorescence measurements of C-DCDHF-DA-labeled cells showed that a 24-h exposure of the labeled cells to $Al(maltol)_3$ caused an increase in fluorescence intensity at 523 nm of the dye incorporated into the cells (Fig. 6A), suggesting that ROS production is associated with $Al(maltol)_3$ treatment of the cells, because it has been reported that C-DCDHF-DA exhibits a broad reactivity to ROS.[30] As shown in Table 2, $Al(maltol)_3$-mediated cell death was effectively protected by pre-treatment with NAC. Furthermore, it was found that NAC also inhibited the onset of the $Al(maltol)_3$ effect against BSO-treated cells (data not shown). From these results, it is suggested that intracellular GSH plays an important role in the protection of cell viability against Al toxicity, because NAC is a precursor of cellular GSH sysnthesis[27] and a potent antioxidant.[31]

There are several studies which show that Al^{3+} has the ability to enhance the pro-oxidant properties of iron.[10—15] We also confirmed that NAC effectively inhibits Fe^{2+}-induced lipid peroxidation of phosphatidylcholine liposomes, assessed by thiobarbituric acid-reactive substances production ($A_{530}=0.320\pm0.002$ and 0.150 ± 0.003 in the systems without and with 1 mM NAC, respectively). From these findings and results, it seems likely that the depletion of intracellular GSH by treatment of PC12 cells with $Al(maltol)_3$ induces lipid peroxidation of the cell membrane lipids *via* the facilitation of ROS production.

On the other hand, lipophilic radical scavengers such as α-tocopherol and BHA did not prevent either $Al(maltol)_3$-induced ROS production or cell death (Figs. 6A, B). As is known, α-tocopherol and BHA act as radical chain blockers by scavenging hydroxyl and lipid peroxyl radicals generated in the membrane lipid layers.[32,33] From these results, it is suggested that intracellular ROS production may be an important mechanism of the onset of Al toxicity. In addition, the lack of prevention of $Al(maltol)_3$-induced ROS production or cell death by the addition of DFO indicates that iron-mediated extracellular Fe^{2+}-induced ROS production and/or lipid peroxidation of the membrane lipids are not directly involved in the onset of $Al(maltol)_3$-induced cell death.

Oxidative stress associated with the generation of free radicals and/or ROS, including superoxide anion and hydroxyl radicals, is now accepted as a common mediator of neurogenerative disorders.[34—36] These free radicals may attack membrane lipids, proteins and nucleic acids to cause cell damage or death.[37] In addition, it has been reported that secondary breakdown products of lipid hydroperoxides, such as 4-hydroxyalkenals, 2-alkenals and malonaldehyde, appear to perturb cell membrane integrity and contribute to the etiology of a number of chronic diseases, including neurodegenerative conditions.[38] Recent experimental evidence indicates that these breakdown products of lipid hydroperoxides easily react with basic amino acid residues in proteins, affecting enzyme activities.[38] We have recently reported that the Na^+/K^+ATPase activity of bovine synaptosomal and reconstituted membranes is markedly inhibited by interaction of the membrane constituents with 4-hydroxy-2-nonenal.[39,40] These findings also pose the possibility that the breakdown products of lipid hydroperoxides are related to the onset mechanism of Al toxicity.

The present paper is the first report showing the involvement of intracellular, not extracellular, ROS production *via* the lowering of the GSH level in Al toxicity development mechanisms. Recently, there have been a number of findings supporting that intracellular ROS production, including superoxide anion radicals and/or hydroxyl radicals, is closely related to the development of neurological disorders.[34—36] Although further detailed studies are necessary to know the exact mechanisms, it seems that these data give us an important clue for analyzing mechanisms concerning the onset of Al-toxicity.

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REFERENCES

1) Perl D. P., Brody A. R., *Science*, **208**, 297—299 (1980).
2) Harrington C. R., Wischik C. M., McArtur F. K., Taylor G. A., Edwardson J. A., Candy J. M., *Lancet*, **343**, 993—997 (1994).
3) Gajdusek D. C., Salazar A. M., *Neurology*, **32**, 107—126 (1982).
4) Perl D. P., Gadjusek C. D., Garruto R. M., Yanagihara R. T., Gibbs C. J., Jr., *Science*, **217**, 1053—1055 (1982).
5) Suarez-Fernandez M. B., Soldado A. B., Sanz-Medel A., Vega J.-A., Novelli A., Fernandez-Sanchez M. T., *Brain Res.*, **835**, 125—136 (1999).
6) Ohyashiki T., Satoh E., Okada M., Takadera T., Sahara M., *Toxicology*, **176**, 195—207 (2002).
7) Savory J., Huang Y., Herman M. M., Reyes M., Wills M. R., *Brain Res.*, **669**, 325—329 (1995).
8) Campbell A., Prasad K. N., Bondy S. C., *Free Rad. Biol. Med.*, **26**, 1166—1171 (1999).
9) Gutteridge J. M. C., Quinlan G. J., Clark I., Halliwell B., *Biochim. Biophys. Acta*, **835**, 441—447 (1985).
10) Ohyashiki T., Karino T., Matsui K., *Biochim. Biophys. Acta*, **1170**, 182—188 (1993).
11) Ohyashiki T., Suzuki S., Satoh E., Uemori Y., *Biochim. Biophys. Acta*, **1389**, 141—149 (1998).
12) Oteiza P. I., Fraga C. G., Keen C. L., *Arch. Biochem. Biophys.*, **300**, 517—521 (1993).
13) Oteiza P. I., *Arch. Biochem. Biophys.*, **308**, 374—379 (1994).
14) Xie C. X., Mattson M. P., Lovell M. A., Yokel R. A., *Brain Res.*, **743**, 271—277 (1996).
15) Ohyashiki T., Nunomura M., *Biochim. Biophys. Acta*, **1484,** 241—250 (2000).
16) Reed D. J., *Annu. Rev. Pharmacol. Toxicol.*, **30**, 603—631 (1990).
17) Meister A., Anderson M. E., *Annu. Rev. Biochem.*, **52**, 711—760 (1983).
18) Hjelle O. P., Chaudhry F. A., Ottersen O. P., *Eur. J. Neurisci.*, **6,** 793—804 (1994).
19) Langeveld C. H., Schepens E., Jongenelen C. A. M., Stoof J. C., Hjelle O. P., Ottersen O. P., Drukarch B., *Neuroreport*, **7**, 1833—1836 (1996).
20) Makar T. K., Nedergaad M., Preuss A. S., Gelbhard A. S., Perumal A. S., Cooper A. J. L., *J. Neurochem.*, **62**, 45—53 (1994).
21) Tan S., Wood M., Maher P., *J. Neurochem.*, **71**, 95—105 (1998).
22) Ishige K., Schubert D., Sagara Y., *Free Radic. Biol. Med.*, **30**, 433—446 (2001).
23) Finnegan M. M., Rettig S. J., Orvig C., *J. Am. Chem. Soc.*, **108**, 5033—5035 (1986).
24) Shrieve D. C., Bump E. A., Rice G. C., *Biochem. J.*, **263**, 14107—14114 (1988).
25) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
26) Griffth O. W., Meister A., *J. Biol. Chem.*, **254**, 7558—7560 (1979).
27) De Vries N., De Flora S., *J. Cell Biochem*. (Suppl. F17), 270—277 (1993).
28) Ghibelli L., Coppola S., Rotilio G., Lafavia E., Maresca V., Ciriolo M. R., *Biochem. Biophys. Res. Commun.*, **216**, 313—320 (1995).
29) Rollet-Labelle E., Grange M.-J., Elbim C., Marquetty C., Gougerot-Pocidalo M.-A., Pasquier C., *Free Radic. Biol. Med.*, **24**, 563—572 (1998).

30) Satoh T., Sakai N., Enokido Y., Uchiyama Y., Hatanaka H., *J. Biochem.* (Tokyo), **120**, 540—546 (1996).
31) Ito, K., Nakazato, T., Yamato, K., Miyakawa, Y., Yamada, T., Hozumi, N., Segawa, K., Ikeda, Y., Kizaki, M., Cancer Res., 64, 1071—1078 (2004).
32) Gotoh N., Shimizu K., Komuro, E., Tsuchiya J., Noguchi N., Niki E., *Biochim. Biophys. Acta*, **1128**, 147—154 (1992).
33) Bors W., Michel C., Saran M., *Bull. Eur. Physiopathol. Respir.,* **17***,* (Suppl.), 13—18 (1981).
34) Ames B. N., Shigenaga M. K., Hagen T. M., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 7915—7922 (1993).
35) Coyle J. T., Puttfarcken P., *Science*, **262**, 689—695 (1993).
36) Sies H., *Exp. Physiol.*, **82**, 291—295 (1997).
37) Halliwell B., *J. Neurochem.*, **59**, 1609—1623 (1992).
38) Esterbauer H., Schaur R. J., Zollner H., *Free Radic. Biol. Med.*, **11**, 81—128 (1991).
39) Kadoya A., Miyake H., Ohyashiki T., *Biol. Pharm. Chem.*, **26**, 787—793 (2003).
40) Miyake H., Kadoya A., Ohyashiki T., *Biol. Pharm. Chem.*, **26**, 1652—1656 (2003).