Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 68 (2004) 659–666
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Differential response of MG132 cytotoxicity against small cell lung cancer cells to changes in cellular GSH contents

Jang Ho Bang[a], Eun Sook Han[a], Inja Lim[b], Chung Soo Lee[a,*]

[a]Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, South Korea
[b]Department of Physiology, College of Medicine, Chung-Ang University, Seoul 156-756, South Korea

Received 16 March 2004; accepted 21 April 2004

Abstract

The effect of the depletion or oxidation of cellular GSH on cytotoxicity of MG132 was assessed. Viability loss and decrease in GSH contents in small cell lung cancer (SCLC) cells treated with MG132 was attenuated by caspase inhibitors (z-IETD.fmk, z-LEHD.fmk and z-DQMD.fmk). Thiol compounds (*N*-acetylcysteine and *N*-(2-mercaptopropionyl)glycine) and free radical scavengers reduced MG132-induced cell death. Antioxidants, including *N*-acetylcysteine, inhibited the MG132-induced nuclear damage, loss in mitochondrial transmembrane potential, cytosolic accumulation of cytochrome *c* and caspase-3 activation. Depletion of GSH due to buthionine sulfoxime did not affect the cell viability loss, ROS formation and GSH depletion due to MG132 in SCLC cells. A thiol oxidant monochloramine, *p*-chloromercuribenzoate and *N*-ethylmaleiamide also did not affect cytotoxicity of MG132. The results suggest that the toxicity of MG132 on SCLC cells is mediated by activation of caspase-8, -9 and -3. Removal of free radicals and recovery of GSH contents may attenuate MG132-induced apoptotic cell death. Nevertheless, depletion or oxidation of cellular GSH may not affect toxicity of MG132.
© 2004 Elsevier Inc. All rights reserved.

Keywords: MG132; Cellular GSH level; Small cell lung cancer cells; Mitochondrial membrane permeability; Cell injury; Differential effect

1. Introduction

Enhanced oxidative stress and defects in mitochondrial function are involved in the induction of necrotic or apoptotic cell death [1]. The membrane permeability transition of mitochondria is recognized as a central event in the course of toxic and oxidative forms of cell injury [2,3]. Opening of the mitochondrial permeability transition pore causes a depolarization of the transmembrane potential, releases of Ca^{2+} and cytochrome *c* and loss of oxidative phosphorylation, which results in loss of cell viability. The ubiquitin/proteasome pathway, responsible for mediating the majority of intracellular proteolysis, plays a crucial role in the regulation of many normal cellular processes, including the cell cycle, differentiation and apoptosis [4–6]. Apoptosis in cancer cells is closely connected with the activity of ubiquitin/proteasome pathway [7,8]. Proteasome inhibitors have been shown to induce apoptotic cell death through formation of ROS [9,10].

The ROS formation and GSH depletion due to toxic substances may cause mitochondrial dysfunction, leading to cell viability loss [1,11]. The mitochondrial membrane permeability could be affected by the redox state of dithiols [12]. The redox state of cellular GSH is an important modulatory element in the protein ubiquitination pathway [13]. The ROS formation and GSH depletion due to proteasome inhibitors may cause mitochondrial dysfunction and subsequent cytochrome *c* release, which leads to cell viability loss [10,14]. *N*-Acetylcysteine, a thiol compound, is demonstrated to reduce the proteasome inhibitor-induced apoptotic cell death [10,15,16]. Nevertheless, it

Abbreviations: SCLC, small cell lung cancer; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; $DCFH_2$-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescin; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; MPG, *N*-(2-mercaptopropionyl)glycine; GSH, glutathione; GSSG, oxidized glutathione; MTT, 1, 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide ; z-DQMD.fmk, z-Asp-(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone; z-IETD.fmk, z-Ile-Glu-(O-ME)-Thr-Asp(O-Me) fluoromethyl ketone; z-LEHD.fmk, z-Leu-Glu-(O-ME)-His-Asp(O-Me) fluoromethyl ketone; ROS, reactive oxygen species

* Corresponding author. Tel.: +82 2 820 5659; fax: +82 2 815 3856.
E-mail address: leecs@cau.ac.kr (C.S. Lee).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.04.010

is uncertain whether cytotoxicity of proteasome inhibitor is modulated by the depletion or oxidation of cellular GSH.

MG132 is a peptide aldehyde, which effectively blocks the proteolytic activity of 26S proteasome complex, behaving as a potent inhibitor of the chymotryptic-like activity [17]. MG132 causes apoptotic cell death through formation of ROS [18,19]. However, the effect of GSH depletion against mitochondrial damage due to MG132 has not been clarified. The purpose of the present study was to explore the influence of the depletion or oxidation of cellular GSH against the toxicity of MG132 on SCLC cells in relation to the mitochondrial membrane permeability and apoptotic cell death. We examined the effects of L-buthionine sulfoximine (a selective inhibitor of γ-glutamylcysteine synthetase), monochloramine (a thiol oxidant), *p*-chloromercuribenzoate (an inhibitor of cell surface thiol groups) and *N*-ethylmaleiamide (an inhibitor of cell surface and cytosol thiol groups) on cytotoxicity of MG132.

2. Materials and methods

2.1. Materials

z-DQMD.fmk and z-IETD.fmk were purchased from Calbiochem-Novabiochem Co., TiterTACS™ colorimetric apoptosis detection kit was from Trevigen, Inc., Quantikine® M human cytochrome *c* assay kit was from R&D systems and ApoAlert™ CPP32/Caspase-3 assay kit was from CLONTECH Laboratories Inc. MG132, *N*-acetylcysteine, MPG, carboxy-PTIO, z-LEHD.fmk, H33258, $DiOC_6(3)$, $DCFH_2$-DA, MTT and other chemicals were purchased from Sigma-Aldrich Inc.

2.2. Preparation of monochloramine

NH_2Cl was prepared freshly on the day of use by the NaOCl-induced oxidation of ammonium chloride [20]. One volume of NaOCl was mixed with 4 vol. of 20 mM NH_4Cl in 10 mM K_2HPO_4 buffer, pH 8.0 at 4 °C. The concentration of NH_2Cl was determined using a molar extinction coefficient of 42.9 at 242 nm.

2.3. Culture of small lung cancer cells

The human small cell lung cancer cells (SCLC, NCI-H889) were obtained from the Korean cell line bank. SCLC cells were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 atmosphere at 37 °C. The culture medium was changed every 3 days, and the cells were subcultured about once a week. Cells (1×10^7) were plated on polystyrene 60 × 15 mm cell culture dishes 48–72 h before experiments. Cells were washed with RPMI containing 1% FBS and replated onto 96-well plates at a density of 4×10^4 cells per well in a volume of 200 μl (or varying numbers of cells/ml in 24-well plates). Cells were treated with MG132 in RPMI containing 1% FBS for 24 h at 37 °C.

2.4. Cell viability assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [21]. SCLC cells (4×10^4) were treated with MG132 for 24 h at 37 °C, and then were incubated with 10 μl of 10 mg/ml MTT solution for 2 h at 37 °C. Culture medium was removed, and dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Spectra MAX 340). Cell viability was expressed as a percentage of the value in control cultures.

2.5. Morphological observation of nuclear change

SCLC cells (1×10^6 cells/ml) were treated with MG132 for 24 h at 37 °C, and the nuclear morphological change was assessed using Hoechst dye 33258 [22]. SCLC cells were incubated with 1 μg/ml Hoechst 33258 for 3 min at room temperature, and nuclei were visualized using an Olympus Microscope with a WU excitation filter.

2.6. Measurement of apoptosis in cells

Apoptosis was assessed by measuring the DNA fragmentation, which occurs following the activation of endonucleases. SCLC cells (1×10^5 cells/ml) were treated with MG132 for 24 h at 37 °C. Cells were washed with phosphate buffered saline (PBS) and fixed with formaldehyde solution. Nucleotide (dNTP) was incorporated at the 3′-ends of DNA fragments using terminal deoxynucleotidyl transferase (TdT). This nucleotide was detected using a horseradish-peroxidase and TACS-Sapphire according to TiterTACS protocol. Data were expressed as absorbance at 450 nm.

2.7. Flow cytometric measurement of mitochondrial transmembrane potential

Changes in the mitochondrial transmembrane potential during the MG132-induced apoptosis in SCLC cells were quantified by flow cytometry with the cationic lipophilic dye $DiOC_6(3)$ [23]. Cells (1×10^6 ml^{-1}) were treated with MG132 for 4 h at 37 °C. $DiOC_6(3)$ (40 nM) was added to the medium, and incubation was performed for 15 min at 37 °C. After centrifugation at 1500 × *g* for 5 min, the supernatants were removed, and the pellets were resuspended in PBS containing 0.5 mM EDTA. For analysis, a FACScan cytofluorometer (Becton Dickinson)

with argon laser excitation at 501 nm was used to assess 10,000 cells from each sample.

2.8. Measurement of cytochrome c release

The release of cytochrome *c* from mitochondria into the cytosol was assessed by using a solid phase ELISA kit for the detection of human cytochrome *c*. SCLC cells (5×10^5 ml^{-1}) were harvested by centrifugation at 800 × *g* for 10 min, washed twice with PBS and resuspended in solution (in mM): 250 sucrose, 20 HEPES-KOH (pH 7.5), 10 KCl, 1.5 $MgCl_2$, 1 EDTA, 1 EGTA, 0.5 dithiothreitol and 0.1 phenylmethylsulfonylfluoride. Cells were further homogenized by successive passages through a 26-G hypodermic needle. The homogenates were centrifuged at 100,000 × *g* for 30 min, and the supernatant was used for analysis of cytochrome *c*. Supernatants were added into the 96-well microplates that contain cytochrome *c* conjugate and are coated with monoclonal antibody specific for human cytochrome *c*. Absorbance of samples was measured at 450 nm in a microplate reader. A standard curve was constructed by adding diluted solutions of cytochrome *c* standard, handled like samples, to the microplates coated with monoclonal antibody. The amount was expressed as nanograms/ml by reference to the standard curve.

2.9. Measurement of caspase-3 activity

Apoptosis in SCLC cells was assessed by measuring the activity of caspase-3, an apoptotic factor [24]. Cells (2×10^6 cells/ml) were treated with MG132 for 24 h at 37 °C, and the caspase-3 activation was determined as described in user's manual of the ApoAlert™ CPP32/Caspase-3 assay kit. The supernatant obtained by centrifugation of lysed cells was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) and was incubated for 1 h at 37 °C. Absorbance of the chromophore *p*-nitroanilide produced was measured at 405 nm. The standard curves were obtained from absorbance of the *p*-nitroanilide standard reagent diluted with cell lysis buffer (up to 20 nM). One unit of the enzyme was defined as the activity producing 1 nmol of chromophore *p*-nitroanilide.

2.10. Measurement of intracellular ROS formation

The dye $DCFH_2$-DA, which is oxidized to fluorescent DCF by hydroperoxides, was used to measure relative levels of cellular peroxides [25]. SCLC cells (4×10^4) were treated with MG132 for 24 h at 37 °C, washed and suspended in FBS-free RPMI. Cells were incubated with 50 μM dye for 30 min at 37 °C and washed with RPMI. The cell suspensions were centrifuged at 412 × *g* for 10 min, and medium was removed. Cells were lysed with 1% Triton X-100, and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (SPECTRAFLUOR, TECAN).

2.11. Measurement of total glutathione

The total glutathione (reduced form GSH plus oxidized form GSSG) was determined using glutathione reductase [26]. SCLC cells (4×10^4) were treated with MG132 for 24 h at 37 °C, centrifuged at 412 × *g* for 10 min in a microplate centrifuge, and medium was removed. Cells were lysed with 2% 5-sulfosalicylic acid (100 μl) and then incubated in 100 μl of the reaction mixture containing 22 mM sodium EDTA, 600 μM NADPH, 12 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) and 105 mM NaH_2PO_4, pH 7.5 at 37 °C Glutathione reductase (20 μl, 100 U/ml) was added, and the mixture further incubated for 10 min. Absorbance was measured at 412 nm using a microplate reader. The standard curve was obtained from absorbance of the diluted commercial GSH incubated in the mixture as in samples.

2.12. Measurement of oxidized glutathione

SCLC cells (4×10^4) were treated with MG132 for 24 h at 37 °C, 5-sulfosalicylic acid added to dissolve cells and centrifuged at 412 × *g* for 10 min. The supernatants were used for analysis of GSSG [27]. Supernatants (100 μl) were reacted with 40 μl of 40 mM *N*-ethylmaleimide for 30 min at room temperature, and to this mixture 0.86 ml of 0.1N NaOH was added. This mixture (100 μl) was mixed with 1.8 ml of 0.1N NaOH solution and then were reacted with 100 μl of *o*-phthalaldehyde (final concentration, 500 μg) for 15 min at room temperature. The amounts of GSSG were measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm using a fluorescence microplate reader. The standard curve was obtained from absorbance of the diluted commercial GSSG incubated in the mixture as in samples.

2.13. Statistical analysis

Data are expressed as means ± S.E.M. Data were analysed using one-way ANOVA. When significance was detected, post hoc comparisons between the different groups was made using Duncan's test for multiple comparisons. A probability less than 0.05 was considered to be statistically significant.

3. Results

3.1. Cell death and decrease in GSH contents due to MG132

Human SCLC cells treated with 15 μM MG132 for 24 h showed cell death and decrease in the GSH contents by 60

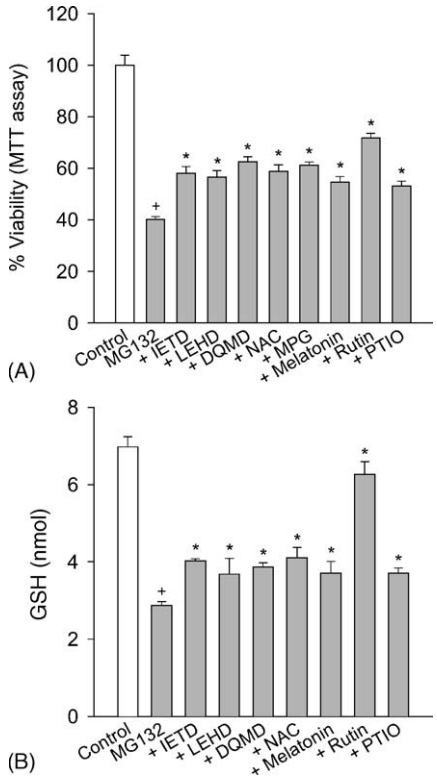

Fig. 1. Cell death and decrease in GSH contents due to MG132. SCLC cells were treated with 15 μM MG132 in the presence of caspase inhibitors [40 μM of z-IETD.fmk (IETD), z-LEHD.fmk (LEHD) and z-DQMD.fmk (DQMD)] or various antioxidants [1 mM *N*-acetylcysteine (NAC), 1 mM MPG, 100 μM melatonin, 50 μM rutin and 25 μM carboxy-PTIO (PTIO)] for 24 h at 37 °C. After the treatment, cell viability (A) and total GSH contents (B) were determined. Data represent means ± S.E.M. ($N = 6$), $^{+}P < 0.05$ compared with control (percentage of control); and $^{*}P < 0.05$ compared with MG132 alone.

and 59%, respectively (Fig. 1). MG132-induced cell death was inhibited by z-IETD.fmk (a cell permeable inhibitor of caspase-8), z-LEHD.fmk (a cell permeable inhibitor of caspase-9) or z-DQMD.fmk (a cell permeable inhibitor of caspase-3) (each 40 μM). Treatment with 1 mM thiol compounds (*N*-acetylcysteine and MPG), 100 μM melatonin (a scavenger of reactive oxygen and nitrogen species), 50 μM rutin (the scavenger of nitric oxide and inhibitor of lipid peroxidation) and 25 μM carboxy-PTIO (a scavenger of nitric oxide) decreased MG132-induced cell death (Fig. 1A). Decrease in cellular GSH levels has been shown to increase the sensitivity of cells to damaging effect of various oxidants [11]. Like the effect on cell death, the specific caspase inhibitors (z-IETD.fmk, z-LEHD.fmk and z-DQMD.fmk) and antioxidants (*N*-acetylcysteine, melatonin, rutin and carboxy-PTIO) interfered with the decrease in GSH contents due MG132 (Fig. 1B).

The inhibitory effect of antioxidants suggests that cytotoxicity of MG132 is mediated by oxidative stress. We investigated changes in the ROS formation and GSH contents with time in SCLC cells treated with MG132. The production of ROS within cells was determined by monitoring conversion of $DCFH_2$-DA to DCF. SCLC cells

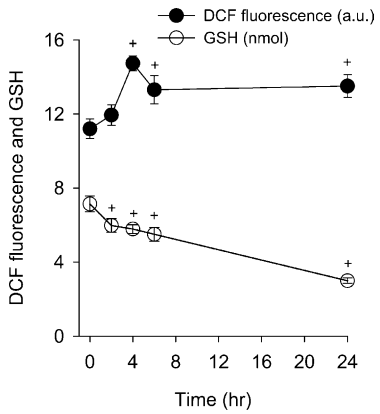

Fig. 2. Change in ROS formation and GSH depletion with time in cells treated with MG132. SCLC cells were treated with 15 μM MG132 for the stated times at 37 °C, and then the ROS formed and GSH contents were determined. The values are expressed as arbitrary units of fluorescence in ROS formation and nmol in GSH contents. Data represent means ± S.E.M. ($N = 6$), $^{+}P < 0.05$ compared with control.

treated with MG132 exhibited a maximum increase in DCF fluorescence at a 4 h-exposure and then showed a low steady level up to 24 h (Fig. 2). Meanwhile, the significant decrease in the GSH contents was detected at a 2 h post addition of MMC (Fig. 2).

3.2. Effect of N-acetylcysteine on MG132-induced nuclear damage

To assess the inhibitory effect of *N*-acetylcysteine against cytotoxicity of MG132 further, we examined its effect on the nuclear morphological changes observed in the MG132-treated cells. Nuclear staining with Hoechst 33258 demonstrated that control SCLC cells had regular and round-shaped nuclei. In contrast, the condensation and

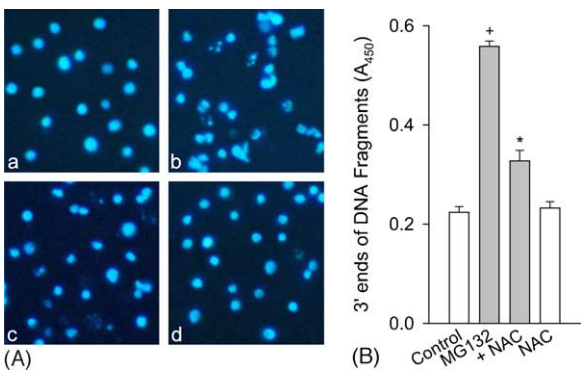

Fig. 3. MG132-induced nuclear damage. SCLC cells were treated with 15 μM MG132 in the presence of 1 mM *N*-acetylcysteine (NAC) for 24 h at 37 °C. In the experiment A, cells were observed by fluorescence microscopy after nuclei staining with Hoechst 33258. Figure represents microscopic morphology of the control cells (a), cells treated with MG132 alone (b), cells treated with MG132 and *N*-acetylcysteine (c) and cells treated with *N*-acetylcysteine alone (d). a–d are representative of four different experiments. In the experiment B, the 3′ ends of DNA fragments were detected as described in Section 2. Data are expressed as absorbance and represent means ± S.E.M. ($N = 6$), $^{+}P < 0.05$ compared with control; and $^{*}P < 0.05$ compared with MG132 alone.

fragmentation of nuclei, characteristic of apoptotic cells, were evident in SCLC cells treated with 15 μM MG132 for 24 h at 37 °C (Fig. 3A). *N*-Acetylcysteine (1 mM) decreased the MG132-induced nuclear damage, while the nuclear morphology in cells exposed to 1 mM *N*-acetylcysteine alone was similar to that in the control cells.

During the process of apoptosis, DNA fragmentation is caused by activation of endonucleases. To clarify further the protective effect on nuclear damage, we assessed the fragmented DNA by measuring the binding of dNTP to the 3′-ends of DNA fragments and detected it by using quantitative colorimetric assay. SCLC cells (1×10^5 cells/ml) were treated with 15 μM MG132 in the presence of *N*-acetylcysteine for 24 h at 37 °C. Control cells show 0.224 ± 0.012 of absorbance ($N = 6$). Treatment of 15 μM MG132 for 24 h caused about 2.5-fold increase in absorbance (Fig. 3B). *N*-Acetylcysteine (1 mM) depressed the MG132-induced increase in absorbance, while absorbance in cells treated with 1 mM *N*-acetylcysteine alone was not significantly different from that in control cells.

3.3. Mitochondrial membrane potential loss, cytochrome *c* release and caspase-3 activation due to MG132

By investigating the effect on the mitochondrial membrane permeability we assessed the cytotoxic effect of MG132. Opening of the mitochondrial membrane permeability transition pore as an early phenomenon in apoptotic cell death causes a release of cytochrome *c* from mitochondria into the cytosol, leading to the activation of caspases [3]. Changes in the mitochondrial transmembrane potential in SCLC cells treated with MG132 were quantified by flow cytometry using the cationic lipophilic dye $DiOC_6(3)$. Exposure of SCLC cells to 15 μM MG132 for 4 h at 37 °C increased the percentage of cells with

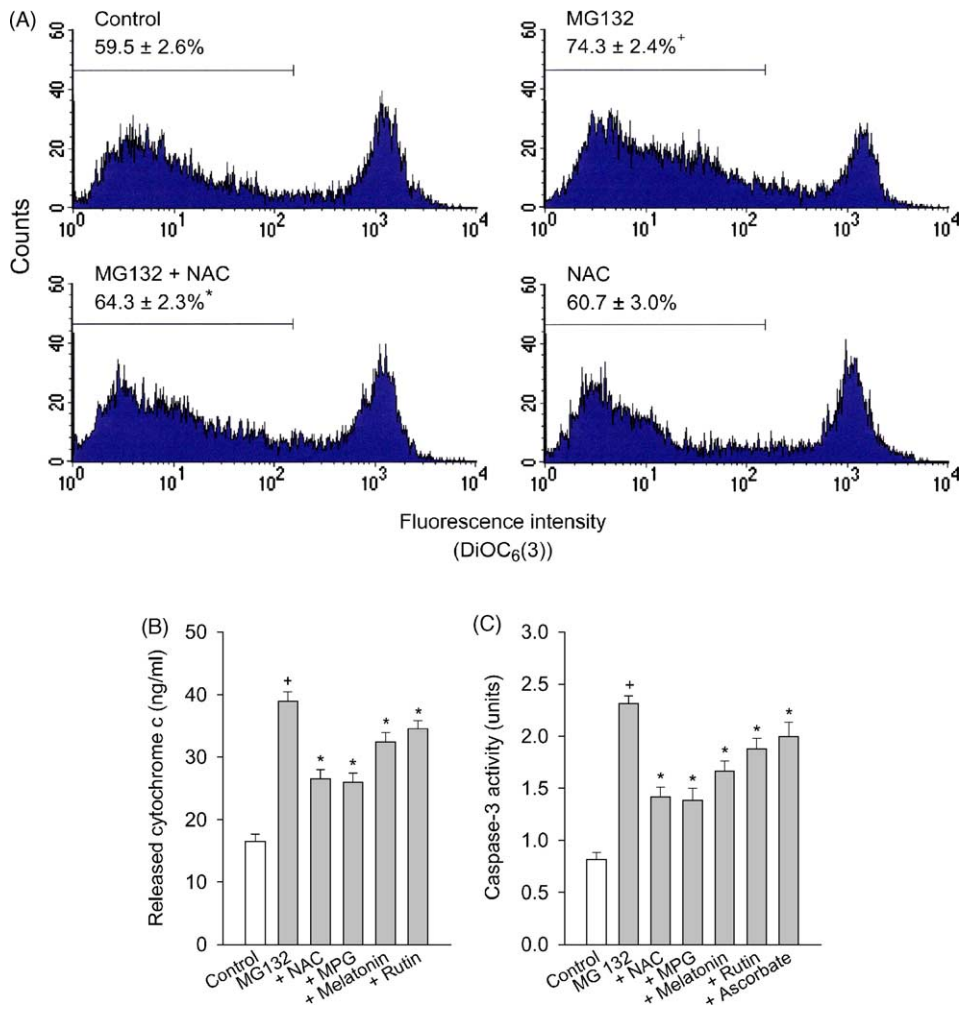

Fig. 4. Loss of the mitochondrial transmembrane potential, release of cytochrome *c* and activation of caspase-3 due to MG132. SCLC cells were treated with 15 μM MG132 in the presence of various antioxidants [1 mM *N*-acetylcysteine (NAC), 1 mM MPG, 100 μM melatonin, 50 μM rutin and 1 mM ascorbate]. In the assay of the mitochondrial transmembrane potential (A), SCLC cells were treated with MG132 for 4 h at 37 °C, and then mixtures were treated with 40 nM $DiOC_6(3)$. In the assay of cytochrome *c* release (B), SCLC cells were treated with MG132 for 4 h at 37 °C, and in the assay of caspase-3 activity (C), cells were treated with MG132 for 24 h. Data represent means ± S.E.M. of ($N = 4$–6), $^{+}P < 0.05$ compared with control; and $^{*}P < 0.05$ compared with MG132 alone.

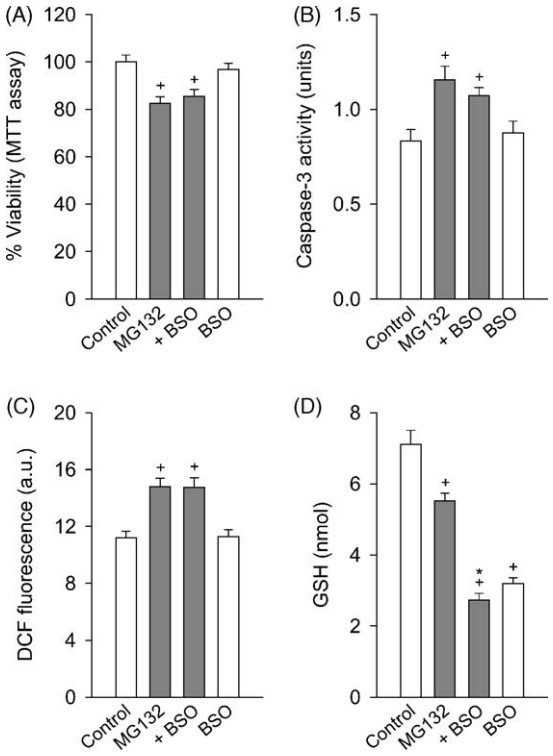

Fig. 5. Effect of GSH depletion on cytotoxicity of MG132. SCLC cells were treated with 500 μM L-buthionine sulfoximine (BSO) for 24 h at 37 °C, and then in this mixture, cells treated with 15 μM MG132 for 6 h at 37 °C. Data represented the percentage of cell viability (A), units in caspase-3 activity (B), arbitrary units of fluorescence in ROS formation (C), and nmol in GSH contents (D). Data represent means ± S.E.M. ($N = 6$), $^{+}P < 0.05$ compared with control; and $^{*}P < 0.05$, compared with MG132 alone.

depolarized mitochondria (characterized by low values of the transmembrane potential). One millimolar *N*-acetylcysteine depressed the MG132-induced increase in depolarized cells, while the transmembrane potential in the presence of *N*-acetylcysteine alone was similar to that in control (Fig. 4A).

MG132-induced cell death was assessed by measuring a release of cytochrome *c* into the cytosol and subsequent activation of caspase-3. Treatment of SCLC cells with 15 μM MG132 for 4 h increased the level of cytochrome *c* in the cytosol significantly (Fig. 4B). Control cells had a caspase-3 activity of 0.82 ± 0.06 U in 2×10^6 cells, while treatment with 15 μM MG132 for 24 h increased caspase-3 activity to 2.31 U (Fig. 4C). The addition of 1 mM *N*-acetylcysteine, 1 mM MPG, 100 μM melatonin, 50 μM rutin and 1 mM ascorbate significantly inhibited the release of cytochrome *c* and caspase-3 activation due to MG132.

3.4. Effect of the depletion or oxidation of cellular GSH on cytotoxicity of MG132

We investigated the effect of the GSH depletion on the cytotoxicity of MG132. SCLC cells were treated with 500 μM L-buthionine sulfoximine, a specific inhibitor of γ-glutamylcysteine synthetase, for 24 h, and then cells were treated with 15 μM MG132 for 6 h. L-Buthionine sulfoximine for a 30 h-treatment decreased the GSH contents in SCLC cells by 55% (Fig. 5D). However, this treatment did not significantly induce the formation of ROS, caspase-3 activation and cell death (Fig. 5A–C). The decrease in GSH contents due to MG132 plus L-buthionine sulfoximine was less than the sum of the each effect of MG132 and L-buthionine sulfoximine. As expected from the results, L-buthionine sulfoximine did not enhance the MG132-induced ROS formation, activation of caspase-3 and cell death (Fig. 5A–C).

Drops in the reduced GSH in cells increase the toxicity of toxic substances, including anticancer drugs [11,28]. The present study examined the effect of oxidizing agent and inhibitor of thiol groups on cytotoxicity of MG132. Monochloramine (NH_2Cl, a thiol oxidant) caused significant cell death and induced formation of GSSG in SCLC cells in a dose-dependent manner (Fig. 6A and B). Nevertheless, NH_2Cl at 50 and 100 μM did not enhance cytotoxicity of MG132, and the MG132 plus NH_2Cl-induced cell death and formation of GSSG was similar to MG132 alone. Cytotoxicity of MG132 in the presence of 200 μM NH_2Cl was less than the sum of the each effect of MG132 and NH_2Cl. Like NH_2Cl, 5 μM of *p*-chloromercuribenzoate (an inhibitor of cell surface thiol groups) and *N*-ethylmaleiamide (an inhibitor of cell surface and cytosol thiol groups) showed a cytotoxic effect on SCLC cells (Fig. 6C). *p*-Chloromercuribenzoate and *N*-ethylmaleiamide did not affect MG132-induced cell death. Cell death due to MG132 in the presence of *p*-chloromercuribenzoate or *N*-ethylmaleiamide was less than the sum of the each effect of both compounds.

4. Discussion

Apoptotic cell death is suggested to be mediated by interaction of ligand with cell surface CD95 receptor, leading to the activation of caspase-8, by mitochondrial dysfunction, results in the release of cytochrome *c* and subsequent activation of caspase-9 and -3 [1,24]. A significant cytotoxic effect of MG132 on SCLC cells was demonstrated by using the MTT assay and by observing nuclear morphological changes and increase in DNA fragments. The condensation and fragmentation of nuclei (Fig. 3) and a significant increase in caspase-3 activity (Fig. 4) were evidence for apoptotic death in SCLC cells following exposure to MG132. The inhibitory effect of specific caspase inhibitors (z-DQMD.fmk, z-IETD.fmk and z-LEHD.fmk) suggests that the MG132-induced apoptotic cell death and depletion of GSH in SCLC cells are mediated by both activation of caspase-8 and mitochondrial damage, leading to the activation of caspase-9 and -3.

Loss of the mitochondrial membrane potential causes the release of cytochrome *c* from mitochondria to the

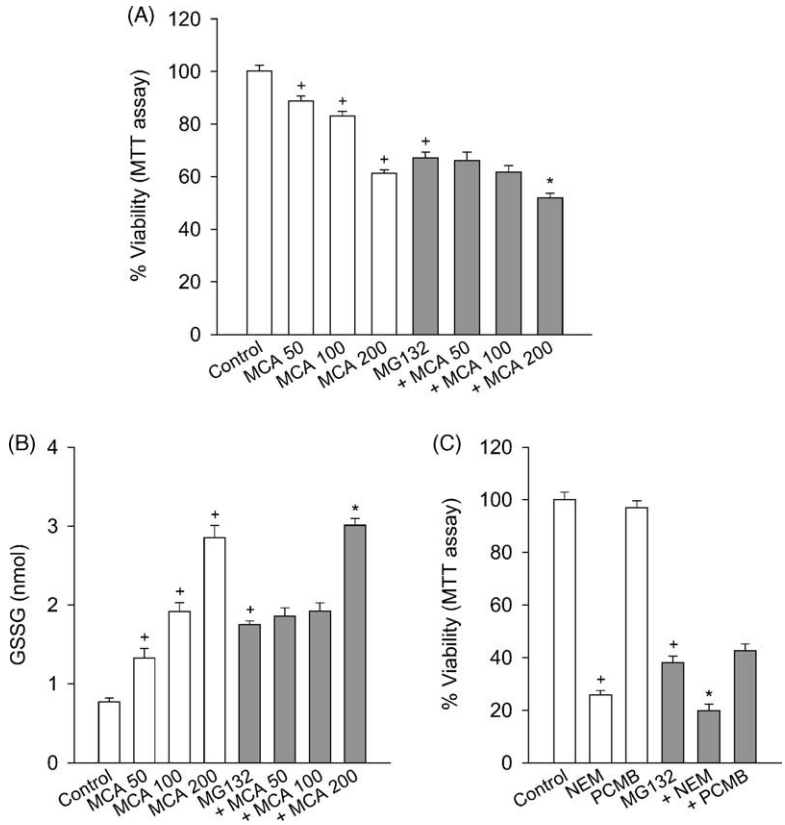

Fig. 6. Effect of the oxidant or inhibitors of thiol groups on MG132-induced cell death. In the experiments A and B, SCLC cells were treated with 15 μM MG132 in the presence of NH_2Cl (MCA, 50–200 μM) for 4 h at 37 °C. In the experiment C, SCLC cells were treated with MG132 in the presence of *p*-chloromercuribenzoate (PCMB) or *N*-ethylmaleiamide (NEM) (5 μM each) for 24 h at 37 °C. Data represent means ± S.E.M. ($N = 6$–10), $^{+}P < 0.05$ compared with control; and $^{*}P < 0.05$ compared with MG132 alone.

cytosol, followed by the activation of caspase-3 that is involved in apoptotic cell death [3]. However, some anticancer drugs cause apoptosis without the cytosolic accumulation of cytochrome *c* [29]. We therefore performed this study to clarify whether the toxicity of MG132 on SCLC cells is mediated by change in the mitochondrial membrane permeability and subsequent release of cytochrome *c*. The present results suggest that the loss of the mitochondrial membrane potential due to MG132 causes the release of cytochrome *c* into the cytosol, which results in activation of caspase-9 and -3. This process may elicit apoptotic cell death. The inhibitory effect of thiol compounds, melatonin, rutin and carboxy-PTIO suggests that MG132 causes viability loss in SCLC cells through the formation of reactive oxygen and nitrogen species and the depletion or oxidation of GSH.

GSH effectively defends cells against ROS and regulates the redox state of many cellular substances [11]. Depletion of mitochondrial GSH has been demonstrated to increase ROS formation [1]. The oxidation of both GSH and NAD(P)H of mitochondria due to oxidative stress has been suggested to induce the mitochondrial membrane permeability transition [12]. Drops in GSH levels and concomitant increase in ROS are found during the glutamate-induced apoptotic process in the immortalized hippocampal cell line, HT22 cell [30]. As the previous report, the present result shows that the GSH contents in SCLC cells treated with MG132 correlated with cell viability loss. The present result suggests that the initial drops of GSH contents due to MG132 may induce increase in the production of ROS.

GSH is known as the main antioxidant system in cells, therefore loss of reduced GSH levels may increase the sensitivity of cells to the damaging effect of toxic substances [11]. The toxicity of anti-cancer drugs may largely depend on the intracellular level of reduced GSH. Depletion of GSH due to L-buthionine-(*S*,*R*)-sulfoximine increases the toxicity of etoposide on K562 human erythroleukemia cells [31] and that of cisplatin on U-937 human promonocytic cells [28]. Mitochondrial dysfunction and cell death due to proteasome inhibitors are reduced by thiol compounds [16,18]. However, the effect of GSH depletion on cytotoxicity of proteasome inhibitors has not been elucidated. The aim of this study was therefore performed to explore whether the cytotoxic effect of MG132 is affected by the depletion or oxidation of cellular GSH. The present results show that despite the depletory effect on the GSH contents, L-buthionine sulfoximine did not enhance the effect of MG132 on the ROS formation, caspase-3 activation and cell death in SCLC cells. Rather, cytotoxicity of MG132 in the presence of L-buthionine sulfoximine was less than the sum of the each effect. Like

no effect of GSH depletion, thiol oxidant NH_2Cl and inhibitors of cell thiol groups (*p*-chloromercuribenzoate and *N*-ethylmaleiamide) did not affect cytotoxicity of MG132 on SCLC cells. The results suggest that the depletion or oxidation of intracellular GSH and the inhibition of surface and cytosol thiol groups do not affect the toxicity of MG132 on SCLC cells.

In conclusion, the results show that MG132 may cause cell death in SCLC cells by inducing the mitochondrial damage, leading to caspase-3 activation, and by activation of caspase-8. The toxicity of MG132 on SCLC cells appears to be prevented by the recovery of GSH contents. Nevertheless, the depletion or oxidation of cellular GSH and the inhibition of thiol groups in cells not appear to affect the toxicity.

References

[1] Chandra J, Samali A, Orrenius S. Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med 2000;29:323–33.

[2] Bernardi P. The permeability transition pore. Control points of a cyclosporine A-sensitive mitochondrial channel involved in cell death. Biochim Biophys Acta 1996;1275:5–9.

[3] Mignotte B, Vayssière JL. Mitochondria and apoptosis. Eur J Biochem 1998;252:1–15.

[4] Fenteany G, Schreiber SL. Lactacystin, proteasome function, and cell fate. J Biol Chem 1998;273:8545–8.

[5] Koepp DM, Harper JW, Elledge SJ. How the cyclin became a cyclin: regulated proteolysis in the cell cycle. Cell 1999;97:431–4.

[6] Orlowski RZ. The role of the ubiquitin-proteasome pathway in apoptosis. Cell Death Differ 1999;6:303–13.

[7] Drexler HCA. Activation of the cell death program by inhibition of proteasome function. Proc Natl Acad Sci USA 1997;94:855–60.

[8] Shah SA, Potter MW, Callery MP. Ubiquitin proteasome pathway: implications and advances in cancer therapy. Surg Oncol 2001;10:43–52.

[9] Lee M, Hyun D-H, Jenner P, Halliwell B. Effect of proteasome inhibition on cellular damage, antioxidant defences and nitric oxide production. J Neurochem 2001;78:32–41.

[10] Ling YH, Liebes L, Zou Y, Perez-Soler R. Reactive oxygen species formation and mitochondrial dysfunction in the apoptotic response to bortezomib, a novel proteasome inhibitor, in human H460 non-small cell lung cancer cells. J Biol Chem 2003;278:33714–23.

[11] Reed DJ. Glutathione: toxicological implications. Annu Rev Pharmacol Toxicol 1990;30:603–31.

[12] Constantini PC, Chernyak BC, Petronilli V, Bernardi P. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. J Biochem Biol 1996;271:6746–51.

[13] Jahngen-Hodge J, Obin MS, Gong X, Shang F, Nowell Jr TR, Gong J, et al. Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. J Biol Chem 1997;272:28218–26.

[14] Qiu JH, Asai A, Chi S, Saito N, Hamada H, Kirino T. Proteasome inhibitors induce cytochrome *c*-caspase-3-like protease-mediated apoptosis in cultured cortical neurons. J Neurosci 2000;20:259–65.

[15] Jha N, Kumar JK, Boonplueang R, Andersen JK. Glutathione decreases in dopaminergic PC12 cells interfere with the ubiquitin protein degradation pathway: relevance for Parkinson's disease? J Neurochem 2002;80:555–61.

[16] Pei XY, Dai Y, Grant S. The proteasome inhibitor bortezomib promotes mitochondrial injury and apoptosis induced by the small molecule Bcl-2 inhibitor HA14-1 in multiple myeloma cells. Leukemia 2003;17:2036–45.

[17] Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol 1998;8:397–403.

[18] Emanuele S, Calvaruso G, Lauricella M, Giuliano M, Bellavia G, D'Anneo A, et al. Apoptosis induced in hepatoblastoma HepG2 cells by the proteasome inhibitor MG132 is associated with hydrogen peroxide production, expression of Bcl-X_s and activation of caspase-3. Int J Oncol 2002;21:857–65.

[19] Wu H-M, Chi K-H, Lin W-W. Proteasome inhibitors stimulate activator protein-1 pathway via reactive oxygen species production. FEBS Lett 2002;526:101–5.

[20] Tamai H, Kachur JF, Grisham MB, Gaginella TS. Scavenging effect of 5-aminosalicylic acid on neutrophil-derived derived oxidants. Possible contribution to the mechanism of action in inflammatory bowel disease. Biochem Pharmacol 1991;41:1001–6.

[21] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.

[22] Oberhammer FA, Pavelka M, Sharma S, Tiefenbacher R, Purchio AF, Bursch W, et al. Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β1. Proc Natl Acad Sci USA 1992;89:5408–12.

[23] Lizard G, Miguet C, Bessede G, Monier S, Gueldry S, Neel D, et al. Impairment with various antioxidants of the loss of mitochondrial transmembrane potential and of the cytosolic release of cytochrome *c* occurring during 7-ketocholesterol-induced apoptosis. Free Radic Biol Med 2000;28:743–53.

[24] Reed JC. Mechanisms of apoptosis. Am J pathol 2000;157:1415–30.

[25] Fu W, Luo H, Parthasarathy S, Mattson MP. Catecholamines potentiate amyloid β-peptide neurotoxicity: involvement of oxidative stress, mitochondrial dysfunction, and perturbed calcium homeostasis. Neurobiol Dis 1998;5:229–43.

[26] van Klaveren RJ, Hoet PHM, Pype JL, Demedts M, Nemery B. Increase in γ-glutamyltransferase by glutathione depletion in rat type II pneumocytes. Free Radic Biol Med 1997;22:525–34.

[27] Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976; 74:214–26.

[28] Troyano A, Fernandez C, Sancho P, de Blas E, Aller P. Effect of glutathione depletion on antitumor drug toxicity (apoptosis and necrosis) in U-937 human promonocytic cells. The role of intracellular oxidation. J Biol Chem 2001;276:47107–15.

[29] Tang DG, Li L, Zhu Z, Joshi B. Apoptosis in the absence of cytochrome *c* accumulation in the cytosol. Biochem Biophys Res Commun 1998;242:380–4.

[30] Tan S, Sagara Y, Liu Y, Maher P, Schubert D. The regulation of reactive oxygen species production during programmed cell death. J Cell Biol 1998;141:1423–32.

[31] Gantchev TG, Hunting DJ. Enhancement of etoposide (VP-16) cytotoxicity by enzymatic and photodynamically induced oxidative stress. Anticancer Drugs 1997;8:164–73.