

The enhancement of propyl gallate-induced apoptosis in HeLa cells by a proteasome inhibitor MG132

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Received October 12, 2010; Accepted December 2, 2010

DOI: 10.3892/or.2010.1121

Abstract. Propyl gallate (PG) used in processed food and medicinal preparations has been shown to induce cell death in normal and cancer cells. The inhibition of proteasome function has emerged as a useful strategy to maneuver apoptosis. Here, we investigated the combined effects of PG and MG132 (a proteasome inhibitor) on HeLa cells in relation to cell growth, cell death, reactive oxygen species (ROS) and glutathione (GSH). PG induced growth inhibition and apoptosis in HeLa cells, accompanied by the loss of mitochondrial membrane potential (MMP; $\Delta\Psi_m$), activation of caspase 3 and PARP cleavage. The levels of ROS and GSH depletion were increased in PG-treated HeLa cells. MG132 intensified apoptosis and PARP cleavage in PG-treated HeLa cells. MG132 also increased ROS levels including mitochondrial $O_2^{\cdot-}$, MMP ($\Delta\Psi_m$) loss and GSH depletion in PG-treated HeLa cells. PG induced a G1 phase arrest of the cell cycle in HeLa cells, which was significantly prevented by MG132. MG132 alone inhibited HeLa cell growth via inducing the cell cycle arrests and triggering apoptosis. Conclusively, the inhibition of proteasome by MG132 plays a role as an enhancement factor in PG-induced apoptosis of HeLa cells via increasing ROS levels and GSH depletion.

Introduction

Propyl gallate (PG) is used as a synthetic antioxidant in processed food and cosmetics to prevent rancidity and spoilage

(1). Because of its ubiquitous usage, the potential toxicity of PG has been investigated to assess various toxicological properties of mutagenicity (2) and cytogenetic effects (3). Despite the unspecified low toxicity of PG, it exerts a variety of effects on tissue and cell functions. Several studies demonstrate the benefits of PG as an antioxidant (4,5) and a chemopreventive agent (6). In contrast, PG can exert prooxidant properties (7,8). It is reported that PG is cytotoxic to isolated rat hepatocytes via mitochondrial dysfunction (9). In addition, PG has been shown to induce cell death in umbilical vein or pulmonary artery endothelial cells (10,11). It is suggested that antioxidative and cytoprotective properties of PG may change to prooxidative, cytotoxic and genotoxic properties in the presence of Cu(II) (12).

The ubiquitin-proteasomal system represents the major non-lysosomal pathway through which intracellular proteins related to cell cycle, differentiation and apoptosis are degraded in eukaryotic cells (13,14). The perturbation of proteasome function has emerged as a useful strategy to maneuver apoptosis. MG132 (carbobenzoxy-Leu-Leu-leucinal) as a peptide aldehyde efficiently blocks the proteolytic activity of proteasome complex (15). Proteasome inhibitors including MG132 have been demonstrated to induce apoptotic cell death through formation of reactive oxygen species (ROS) (16,17). ROS formation and GSH (glutathione) depletion due to proteasome inhibitors may cause mitochondrial dysfunction and subsequent cytochrome c release, which leads to cell viability loss (18,19).

Recently, we demonstrated that PG inhibited the growth of HeLa cells via apoptosis and GSH depletion (20,21) and MG132 inhibited the growth of HeLa cells via cell cycle arrest as well as apoptosis (22). In the present study, we investigated the combined effects of PG and MG132 on HeLa cells in relation to cell growth, cell cycle and apoptosis. Furthermore, we evaluated the changes of ROS and GSH levels in HeLa cells treated with PG and MG132.

Materials and methods

Cell culture. The human cervical HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in a humidified incubator containing 5% CO_2 at 37°C. HeLa cells were cultured in RPMI-1640 (Sigma-Aldrich Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Chemical Co.) and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY).

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Abbreviations: PG, propyl gallate; MG132, carbobenzoxy-Leu-Leu-leucinal; ROS, reactive oxygen species; CDK, cyclin-dependent kinase; SOD, superoxide dismutase; MMP ($\Delta\Psi_m$), mitochondrial membrane potential; FBS, fetal bovine serum; PI, propidium iodide; FITC, fluorescein isothiocyanate; H_2DCFDA , 2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; GSH, glutathione; CMFDA, 5-chloromethylfluorescein diacetate

Key words: propyl gallate, MG132, apoptosis, HeLa, reactive oxygen species, glutathione

Reagents. PG purchased from the Sigma-Aldrich Chemical Co. was dissolved in ethanol at 200 mM as a stock solution. MG132 was purchased from Calbiochem (San Diego, CA). It was dissolved in DMSO solution buffer at 10 mM as a stock solution. Ethanol (0.2%) and DMSO (0.2%) were used as a control vehicle. All stock solutions were wrapped in foil and kept at -20°C .

Cell growth assay. The effect of PG and/or MG132 on HeLa cell growth was determined by trypan blue exclusion cell counting or measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Chemical Co.) dye absorbance of living cells as previously described (23). In brief, 3×10^4 cells per well were seeded in 96-well microtiter plates (Nunc) for cell counting and MTT assays. After exposure to the indicated amounts of PG with or without MG132 for 24 h, cells were collected with trypsin digestion for trypan blue exclusion cell counting or were used for MTT assays. Twenty microliters of MTT solution (2 mg/ml in PBS) was added to each well of the 96-well plates. The plates were incubated for 4 additional hours at 37°C . Media in plates were withdrawn by pipetting, and 200 μl of DMSO was added to each well to solubilize the formazan crystals. Optical density was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunnyvale, CA).

Western blot analysis. The expressions of proteins were evaluated using Western blot analysis, as previously described (24). In brief, 1×10^6 cells were incubated with indicated amounts of PG with or without MG132 for 24 h. The cells were then washed in PBS and suspended in 5 volumes of lysis buffer (20 mM HEPES pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP40, 0.5 mM DTT, 1% protease inhibitor cocktail). Supernatant protein concentrations were determined using the Bradford method. Supernatant samples containing 40 μg total protein were resolved by 8 or 15% SDS-PAGE gels, depending on the sizes of target proteins, transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA) by electroblotting, and then probed with anti-ubiquitin, anti-p27, caspase-3, PARP, catalase, SOD1 and anti- β -actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Blots were developed using an ECL kit (Amersham, Arlington Heights, IL).

Cell cycle analysis. Cell cycle distributions were determined by propidium iodide (PI, Sigma-Aldrich; Ex/Em = 488/617 nm) staining, as previously described (25). In brief, 1×10^6 cells were incubated with the indicated amounts of PG with or without MG132 for 24 h. Cells were then washed with PBS and fixed in 70% ethanol. Cells were washed again with PBS, then incubated with PI (10 $\mu\text{g}/\text{ml}$) with simultaneous RNase treatment at 37°C for 30 min. Cell DNA content was measured using a FACStar flow cytometer (Becton-Dickinson, San Jose, CA).

Annexin V staining. Apoptosis was determined by staining cells with Annexin V-fluorescein isothiocyanate (FITC, PharMingen, San Diego, CA; Ex/Em = 488/519 nm), as previously described (25). In brief, 1×10^6 cells were incubated with the indicated amounts of PG with or without MG132 for

24 h. Cells were washed twice with cold PBS and then resuspended in 500 μl of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2) at a concentration of 1×10^6 cells/ml. Five microliters of Annexin V-FITC was then added to these cells, which were analyzed with a FACStar flow cytometer (Becton-Dickinson).

Measurement of MMP ($\Delta\Psi_m$). MMP ($\Delta\Psi_m$) levels were measured using rhodamine 123 fluorescent dye (Sigma; Ex/Em = 485/535 nm), as previously described (23). In brief, 1×10^6 cells were incubated with the indicated amounts of PG with or without MG132 for 24 h. Cells were washed twice with PBS and incubated with rhodamine 123 (0.1 $\mu\text{g}/\text{ml}$) at 37°C for 30 min. Rhodamine 123 staining intensity was determined by flow cytometry. An absence of rhodamine 123 from cells indicated the loss of MMP ($\Delta\Psi_m$) in cells. The MMP ($\Delta\Psi_m$) levels in the cells, excluding MMP ($\Delta\Psi_m$) loss cells, were expressed as the mean fluorescence intensity (MFI), which was calculated by CellQuest software (Becton-Dickinson).

Detection of intracellular ROS. Intracellular ROS were detected by fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA , Invitrogen Molecular Probes, OR; Ex/Em = 495/529 nm). H_2DCFDA is poorly selective for superoxide anion radical ($\text{O}_2^{\cdot-}$). In contrast, dihydroethidium (DHE; Invitrogen Molecular Probes; Ex/Em = 518/605 nm) is a fluorogenic probe that is highly selective for $\text{O}_2^{\cdot-}$ among ROS. In addition, mitochondrial $\text{O}_2^{\cdot-}$ level was detected by means of MitoSOXTM Red mitochondrial $\text{O}_2^{\cdot-}$ indicator (Invitrogen Molecular Probes; Ex/Em = 510/580 nm) as previously described (26). In brief, 1×10^6 cells were incubated with the indicated amounts of PG with or without MG132 for 1 or 24 h. Cells were then washed in PBS and incubated with 20 μM H_2DCFDA , 20 μM DHE or 5 μM MitoSOXTM Red agent at 37°C for 30 min according to the instructions of the manufacturer. DCF, DHE and MitoSOX fluorescences were detected using a FACStar flow cytometer (Becton-Dickinson). ROS and $\text{O}_2^{\cdot-}$ levels were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software (Becton-Dickinson).

Detection of the intracellular glutathione (GSH). Cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA, Invitrogen Molecular Probes; Ex/Em = 522/595 nm), as previously described (26). In brief, 1×10^6 cells were incubated with the indicated amounts of PG with or without MG132 for 1 or 24 h. Cells were then washed with PBS and incubated with 5 μM CMFDA at 37°C for 30 min. CMF fluorescence intensity was determined using a FACStar flow cytometer (Becton-Dickinson). Negative CMF staining (GSH-depleted) cells were expressed as the percent of (-) CMF cells. The CMF levels in cells, excluding GSH-depleted cells, were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software (Becton-Dickinson).

Statistical analysis. The results represent the mean of at least three independent experiments (mean \pm SD). The data were

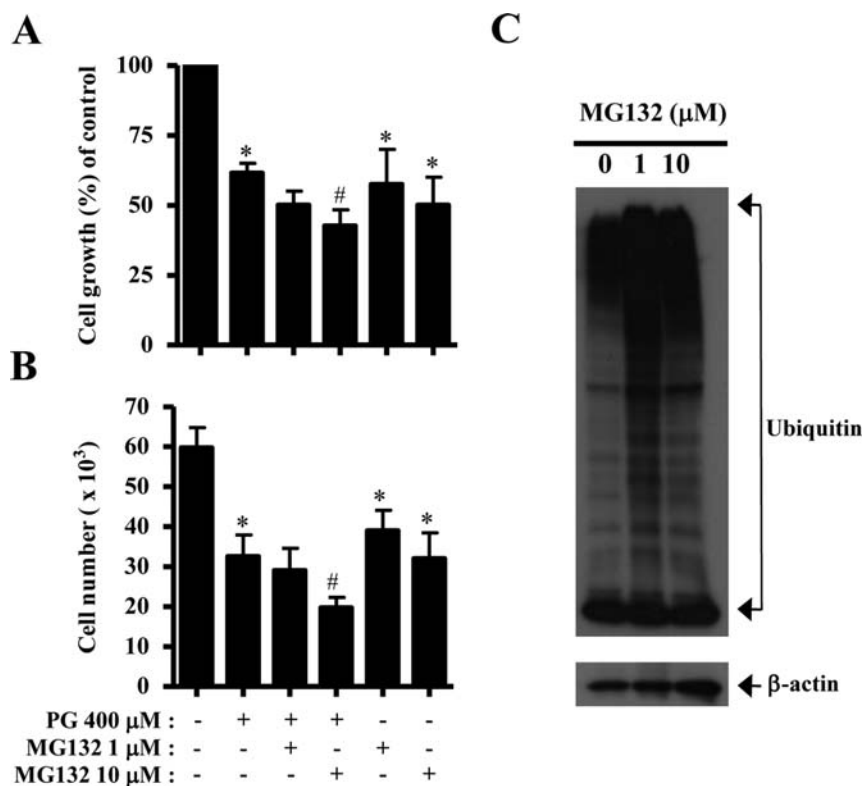


Figure 1. Effects of MG132 on the growth in PG-treated HeLa cells. Exponentially growing cells were treated with PG and/or MG132 for 24 h. Cell growth (A) and cell number (B) were assessed by an MTT assay and trypan blue exclusion cell counting, respectively. (C) 40 μ g samples of protein extracts were resolved by SDS-PAGE gel, transferred onto the PVDF membranes and immunoblotted with the indicated antibodies against ubiquitin and β -actin. * P <0.05 compared with the control group. # P <0.05 compared with cells treated with PG only.

analyzed using Instat software (GraphPad Prism4, San Diego, CA). The Student's t-test or one-way analysis of variance (ANOVA) with post hoc analysis using Tukey's multiple comparison test was used for parametric data. Statistical significance was defined as P <0.05.

Results

Effects of MG132 on cell growth and the cell cycle distribution in PG-treated HeLa cells. For this experiment, the dose of 400 μ M PG was chosen to be a suitable concentration, which inhibited the growth of HeLa cells about 35% using an MTT assay (Fig. 1A) and about 50% using trypan blue cell counting at 24 h (Fig. 1B). MG132 dose-dependently increased the growth inhibition of PG-treated HeLa cells (Fig. 1A and B). MG132 alone inhibited the growth of HeLa control cells (Fig. 1A and B). In addition, we observed a relative increase in anonymous ubiquitinated proteins in MG132-treated HeLa cells (Fig. 1C), suggesting that MG132 inhibits the activity of 26S proteasome complex in HeLa cells. In relation to cell cycle distributions, PG significantly induced a G1 phase arrest of the cell cycle at 24 h (Fig. 2). MG132 significantly inhibited a G1 phase arrest in PG-treated HeLa cells (Fig. 2). Treatment with 1 and 10 μ M MG132 alone significantly arrest G2-M phase and S phase of the cell cycle, respectively (Fig. 2). In addition, p27, a cyclin-dependent kinase inhibitor which typically causes cells to arrest in the G1 phase, was increased in PG-treated HeLa cells, but MG132 completely decreased the p27 protein level (Fig. 2).

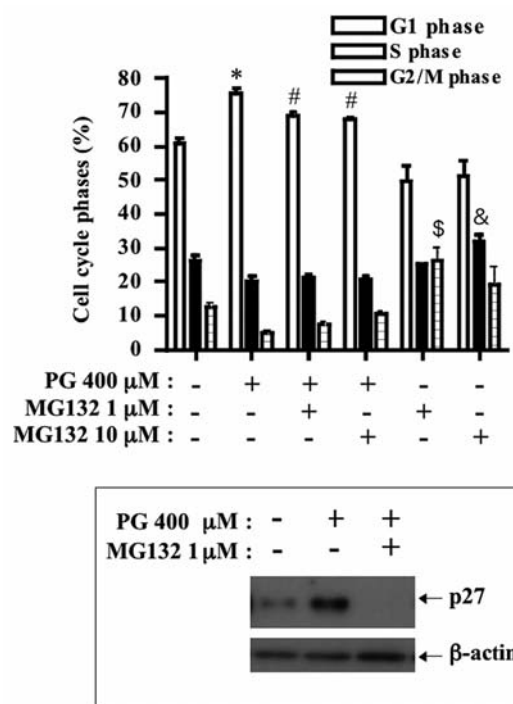


Figure 2. Effects of MG132 on the cell cycle distributions in PG-treated HeLa cells. Exponentially growing cells were treated with PG and/or MG132 for 24 h. The changes of cell cycle phase distribution were assessed by DNA flow cytometric analysis. Graph shows the percents of each phase of the cell cycle. Samples of protein extracts (40 μ g) were resolved by SDS-PAGE gel, transferred onto the PVDF membranes and immunoblotted with the indicated antibodies against p27 and β -actin. * P <0.05 compared with the control group. # P <0.05 compared with cells treated with PG only.

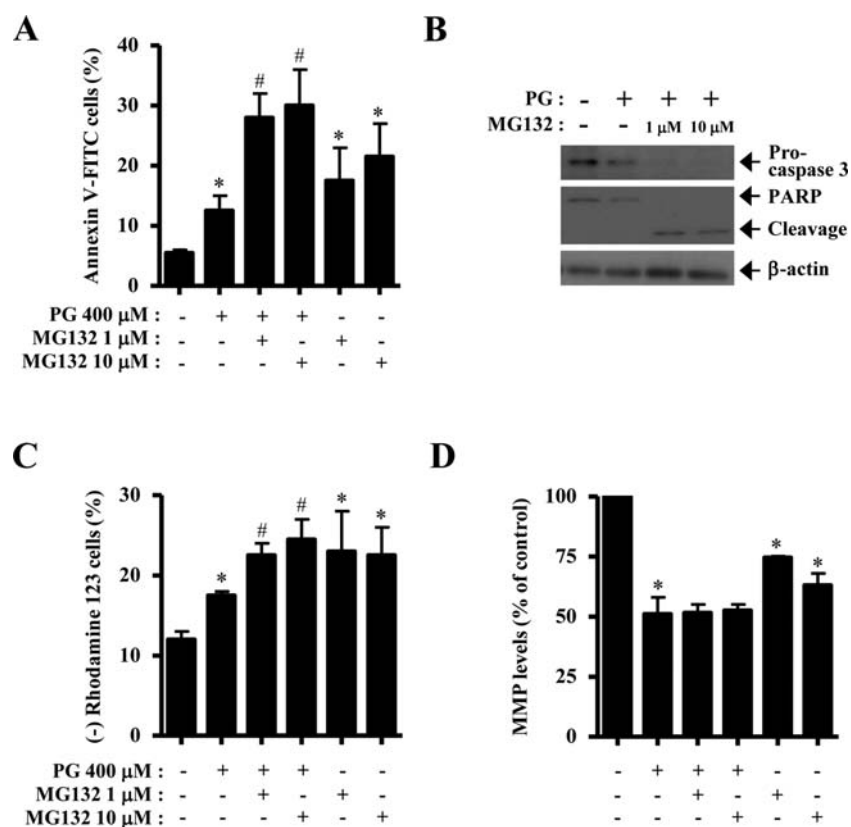


Figure 3. Effects of MG132 on apoptosis and MMP ($\Delta\Psi_m$) in PG-treated HeLa cells. Exponentially growing cells were treated with PG and/or MG132 for 24 h. Annexin V-FITC cells and MMP ($\Delta\Psi_m$) in HeLa cells were measured with a FACStar flow cytometer. (A) Graph shows the percent of Annexin V positive staining cells. (B) 40 μ g samples of protein extracts were resolved by SDS-PAGE gel, transferred onto the PVDF membranes and immunoblotted with the indicated antibodies against caspase-3, PARP and β -actin. (C and D) Graphs show the percent of rhodamine 123 negative [MMP ($\Delta\Psi_m$) loss] cells (C) and MMP ($\Delta\Psi_m$) levels compared with HeLa control cells (D). * $P < 0.05$ compared with the control group. # $P < 0.05$ compared with cells treated with PG only.

Effects of MG132 on apoptosis and MMP ($\Delta\Psi_m$) in PG-treated HeLa cells. PG induced apoptosis in HeLa cells, as evidenced by Annexin V staining (Fig. 3A). MG132 increased the number of Annexin V-FITC positive cells in PG-treated or untreated HeLa cells (Fig. 3A). In relation to apoptosis-related proteins, the level of procaspase-3 was significantly reduced in PG-treated HeLa cells (Fig. 3B), which implied that the activation of caspase-3 occurred in PG-treated cells. MG132 enhanced the decrease of procaspase-3 (Fig. 3B). In addition, cleavage of PARP [poly (ADP-ribose) polymerase] provides one of the most recognizable examples in apoptosis (27). As shown in Fig. 3B, the intact 116 kDa moiety of PARP protein was decreased in PG-treated HeLa cells. MG132 clearly induced the cleavage of PARP in PG-treated HeLa cells (Fig. 3B). In relation to MMP ($\Delta\Psi_m$), the loss of MMP ($\Delta\Psi_m$) was observed in PG-treated cells at 24 h (Fig. 3C). MG132 increased the level of MMP ($\Delta\Psi_m$) loss in PG-treated or untreated HeLa cells (Fig. 3C). In addition, PG reduced MMP ($\Delta\Psi_m$) level [excluding MMP ($\Delta\Psi_m$) loss cells] about 50% compared with control cells (Fig. 3D). MG132 did not affect the MMP ($\Delta\Psi_m$) level in PG-treated cells (Fig. 3D). This agent alone reduced the MMP ($\Delta\Psi_m$) level in HeLa control cells (Fig. 3D).

Effects of MG132 on ROS and $O_2^{\cdot-}$ levels in PG-treated HeLa cells. Intracellular ROS level (as detected by DCF) was

decreased in PG-treated cells at the short time period of 1 h (Fig. 4A) whereas ROS level was increased at 24 h (Fig. 4B). MG132 increased ROS levels in PG-treated and untreated HeLa cells at 1 and 24 h (Fig. 4A and B). Furthermore, PG slightly increased the expression level of catalase in HeLa cells whereas MG132 decreased the level in PG-treated cells (Fig. 4C). PG slightly decreased SOD expression level in HeLa cells and MG132 did not affect the level in PG-treated or untreated HeLa cells (Fig. 4C). Furthermore, red fluorescence derived from DHE reflecting intracellular $O_2^{\cdot-}$ level was increased in PG-treated cells at 1 and 24 h (Fig. 4D and E). MG132 intensified the increased $O_2^{\cdot-}$ levels in PG-treated and untreated cells at 24 h (Fig. 4D and E). In addition, MitoSOX Red fluorescence level, which specifically indicates $O_2^{\cdot-}$ level in the mitochondria, was increased in PG-treated HeLa cells at 24 h (Fig. 4F). MG132 increased the mitochondrial $O_2^{\cdot-}$ levels in PG-treated and untreated HeLa cells (Fig. 4F).

Effects of MG132 on GSH levels in PG-treated HeLa cells. Next, the changes of GSH contents in HeLa cells were determined at 1 or 24 h. PG increased the number of GSH depletion cells about 7% at 24 h compared with HeLa control cells (Fig. 5A and B) but not at 1 h (data not shown). MG132 increased GSH depleted cell numbers in PG-treated and untreated HeLa cells (Fig. 5A and B). In addition, PG did not

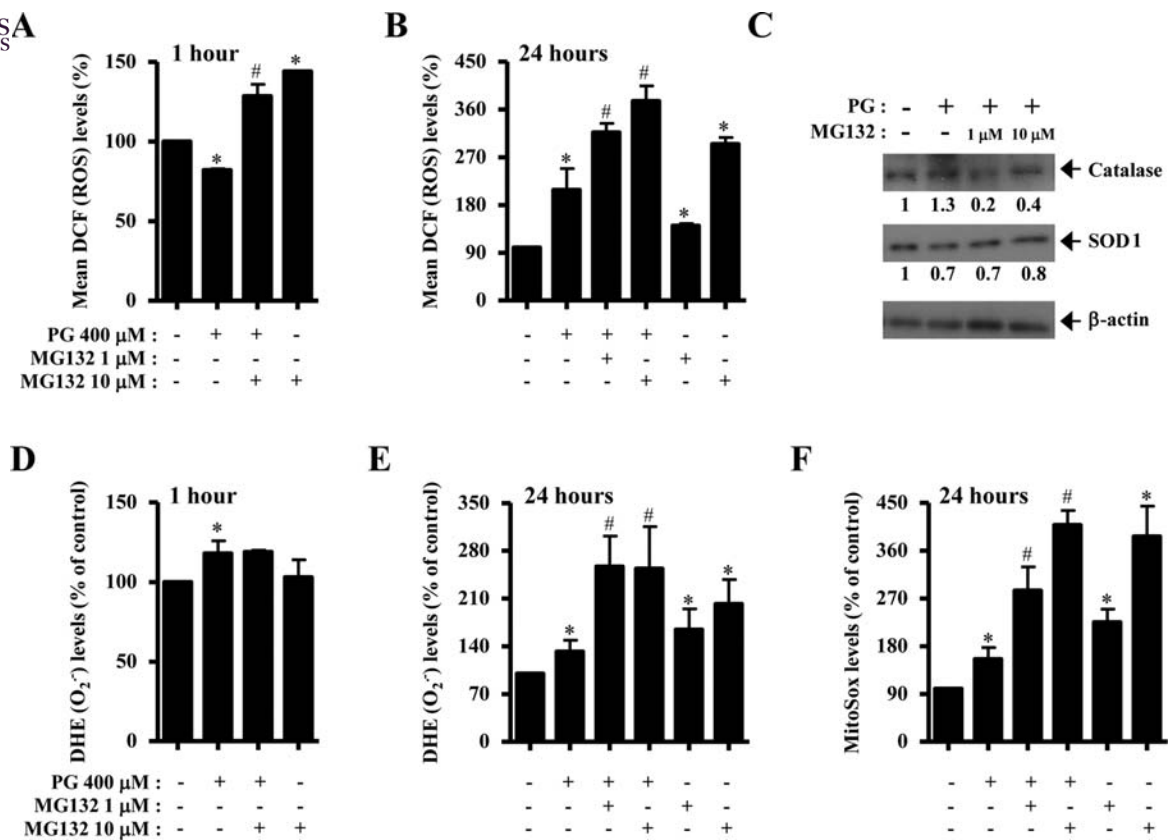


Figure 4. Effects of MG132 on ROS levels in PG-treated HeLa cells. Exponentially growing cells were treated with PG and/or MG132 for 1 or 24 h. ROS levels in HeLa cells were measured using a FACStar flow cytometer. (A and B) Graphs indicate DCF (ROS) levels (%) compared with control cells at 1 h (A) and at 24 h (B). (C) 40 μ g samples of protein extracts were resolved by SDS-PAGE gel, transferred onto the PVDF membranes and immunoblotted with the indicated antibodies against catalase, SOD1 and β -actin. The values under Western blotting indicate the ratio of protein expression levels compared with each β -actin control band. (D and E) Graphs indicate DHE ($O_2^{\cdot-}$) levels (%) compared with control cells at 1 h (D) and 24 h (E). (F) Graph shows the percent of MitoSOx (mitochondrial $O_2^{\cdot-}$) levels compared with control cells at 24 h. * P <0.05 compared with the control group. # P <0.05 compared with cells treated with PG only.

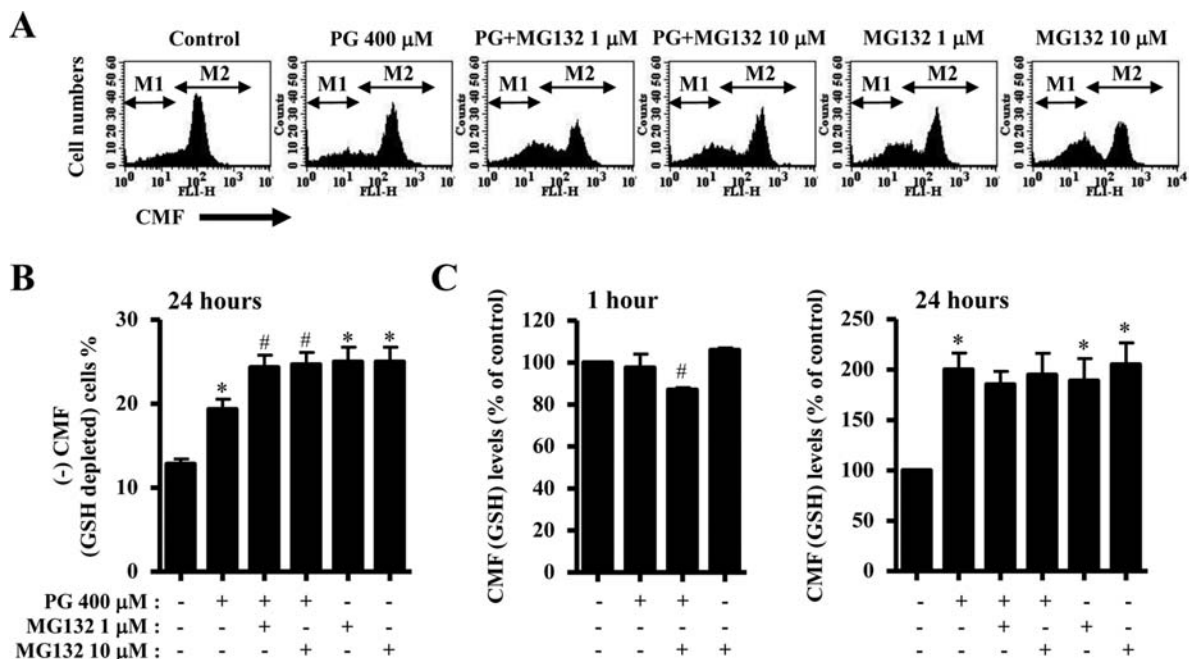


Figure 5. Effects of MG132 on GSH levels in PG-treated HeLa cells. Exponentially growing cells were treated with PG and/or MG132 for 1 or 24 h. GSH levels in HeLa cells were measured using a FACStar flow cytometer. (A) Histograms for CMF intensity in HeLa cells at 24 h. M1 indicates GSH depleted cells. M2 indicates cells excluding GSH depleted cells. (B and C) Graphs show the percent of GSH depleted cells [M1 region in (A)] (B) and mean CMF (GSH) levels [M2 region in (A)] compared with the control group (C). * P <0.05 compared with cells treated with PG only.

significantly change GSH level at 1 h but MG132 decreased GSH level in PG-treated cells (Fig. 5C). At 24 h, PG significantly increased GSH levels (excluding GSH depleted cells) and MG132 did not change the GSH levels (Fig. 5A and C). MG132 alone significantly increased GSH levels in HeLa control cells (Fig. 5A and C).

Discussion

Treatment with MG132 intensified the growth inhibition and apoptosis in PG-treated HeLa cells. MG132 alone significantly induced cell growth inhibition and cell death in HeLa cells. MG132 significantly decreased the number of cells at G1 phase in PG-treated HeLa cells. The proportion of a G1 phase in PG and/or MG132-treated HeLa cells seemed to be affected by the changes of p27 protein levels, which was clearly regulated by these agents. In addition, MG132 increased the level of MMP ($\Delta\Psi_m$) loss in PG-treated cells but did not alter MMP ($\Delta\Psi_m$) level in these cells, implying that MG132 affects MMP ($\Delta\Psi_m$) loss rather than MMP ($\Delta\Psi_m$) level in PG-treated HeLa cells. These data suggest that the inhibition of ubiquitin-proteasomal system by MG132 is involved in cell growth inhibition (or via cell cycle distribution changes) and cell death [or via MMP ($\Delta\Psi_m$) loss] in PG-treated HeLa cells.

Similar to previous reports (20,22), ROS levels including $O_2^{\bullet-}$ levels were increased or decreased in PG-treated HeLa cells depending on the incubation times (1 or 24 h). MG132 also increased the intracellular ROS levels including $O_2^{\bullet-}$ in HeLa control cells. It is reported that PG or MG132 induced cell death by mediating mitochondrial dysfunction (18,28). Likewise, PG or MG132 induced the loss of MMP ($\Delta\Psi_m$) and increased mitochondrial $O_2^{\bullet-}$ levels in HeLa cells. In relation to the effect of MG132 on ROS levels in PG-treated HeLa cells, MG132 enhanced ROS (DCF) levels in these cells. It is possible that this enhancement resulted from the strong down-regulation of catalase expression in PG-treated HeLa cells by MG132. MG132 also increased $O_2^{\bullet-}$ levels including mitochondrial $O_2^{\bullet-}$ in PG-treated HeLa cells without changing SOD expression level. Therefore, it was plausible that the increased $O_2^{\bullet-}$ levels by PG and/or MG132 was due to the mitochondrial dysfunction by these agents. Correctively, these results suggest that the inhibition of proteasome by MG132 plays a role as an enhancement factor in PG-induced apoptosis in HeLa cells by increasing ROS levels including mitochondrial $O_2^{\bullet-}$.

Apoptotic effects are inversely comparative to GSH content (29,30). In addition, it is reported that GSH depletion due to proteasome inhibitors may lead to cell death (18,19). Likewise, PG and/or MG132 increased GSH depleted cell numbers in HeLa cells. It is of note that GSH levels (excluding GSH depleted cells) in PG and/or MG132-treated HeLa cells were increased at 24 h. Probably, these happened to reduce the increasing ROS by these agents. Thus, cells beyond their capacity to resist ROS seemed to be immediately dead. However, PG or MG132 alone did not alter GSH content in HeLa cells at 1 h, and co-treatment with PG and MG132 reduced GSH levels in HeLa cells. These results suggest that PG and/or MG132 differently regulate GSH levels in HeLa cells depending on the incubation times.

In conclusion, the inhibition of proteasome by MG132 plays a role as an enhancement factor in PG-induced apoptosis in HeLa cells via increasing ROS levels and GSH depletion.

Acknowledgements

This study was supported by a grant from the Ministry of Science and Technology (MoST)/Korea Science and Engineering Foundation (KOSEF) through the Diabetes Research Center at Chonbuk National University (2010-0029497) and the National Research Foundation of Korea Grant funded by the Korean Government (MEST) (2010-0021808).

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