The effect of MAPK inhibitors and ROS modulators on cell growth and death of H_2O_2-treated HeLa cells

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Abstract. Reactive oxygen species (ROS) influence the signaling of mitogen-activated protein kinases (MAPKs) involved in cell survival and death. In the present study, the toxicological effect of hydrogen peroxide (H_2O_2) on HeLa cervical cancer cells was evaluated following treatment with MAPK inhibitors [MAP kinase or ERK kinase (MEK), c-Jun N-terminal kinase (JNK) or p38], *N*-acetyl cysteine (NAC) and propyl gallate (PG) (well-known antioxidants), or L-buthionine sulfoximine [BSO; an inhibitor of glutathione (GSH) synthesis]. Treatment with 100 μM H_2O_2 inhibited the growth of HeLa cells and induced cell death, which was accompanied by loss of the mitochondrial membrane potential (MMP; $\Delta\Psi_m$). H_2O_2 did not induce any specific phase arrests of the cell cycle. ROS levels increased, while GSH levels decreased in H_2O_2-treated HeLa cells after 1 and 24 h of treatment. The MAPK inhibitors enhanced H_2O_2-induced HeLa cell death, while only p38 inhibitor increased ROS levels. Both NAC and PG attenuated H_2O_2-induced HeLa cell growth inhibition and death together with the suppression of ROS levels. BSO increased ROS levels in H_2O_2-treated HeLa cells without increasing cell death. The levels of MMP ($\Delta\Psi_m$) loss and GSH depletion were not closely associated with the levels of apoptosis in HeLa cells treated with the MAPK inhibitors, NAC, PG or BSO, in the presence of H_2O_2. In conclusion, H_2O_2 induced HeLa cell growth inhibition and death. MAPK inhibitors generally enhanced H_2O_2-induced HeLa cell death. In particular, p38 inhibitor increased ROS levels in H_2O_2-treated HeLa cells, while NAC and PG attenuated H_2O_2-induced HeLa cell death by suppressing ROS levels.

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Abbreviations: ROS, reactive oxygen species; GSH, glutathione; MAPK, mitogen-activated protein kinase; MEK, MAP kinase or ERK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MMP ($\Delta\Psi_m$), mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; PI, propidium iodide; H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; CMFDA, 5-chloromethylfluorescein diacetate; NAC, *N*-acetyl cysteine; PG, propyl gallate; BSO, L-buthionine sulfoximine

Key words: HeLa cell, hydrogen peroxide, cell death, reactive oxygen species, mitogen-activated protein kinase

Introduction

Reactive oxygen species (ROS) are a group of oxygen moieties that are formed by the incomplete one-electron reduction of oxygen. The major ROS include hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$). Among ROS, H_2O_2 diffuses freely over cell membranes prior to reacting with specific molecular targets due to its solubility in both lipid and aqueous environments and its fairly low reactivity. $O_2^{\bullet-}$ is metabolized to H_2O_2 by superoxide dismutases (1). H_2O_2 is further detoxified to O_2 and H_2O by catalase or glutathione (GSH) (2).

ROS might affect the activity of mitogen-activated protein kinases (MAPKs), which are involved in important signaling pathways in cell proliferation, differentiation and cell death in response to a variety of stimuli (3,4). The decision to proliferate, arrest or die depends on the relative strengths of cell survival and apoptotic signals triggered by ROS. The three main signaling modules of MAPKs are the extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38, which has emerged as an important signaling pathway from the membrane to nucleus (3). Each MAPK pathway has different upstream activators and the activated MAPKs promote differential transcriptional stimulation of multiple genes via the phosphorylation of unambiguous transcription factors (5). MAPKs also sense the cellular redox status and are common targets for ROS. JNK and p38 are mainly activated by ROS or a mild oxidative shift, initiating procedures related to apoptosis (6,7). However, the two kinases differentially affect the levels of apoptosis (8). ROS also provoke or inhibit ERK pathway (9,10). In most cases, ERK activation has been shown to have a pro-survival rather than a pro-apoptotic effect (11). In addition, MAPK pathways are also activated by the direct inhibition of MAPK phosphatases by ROS. Since opposite effects of MAPKs by various ROS can occur in cells,

the association between ROS and MAPKs needs to be further elucidated, particularly signaling pathways related to cell survival and death.

Cervical neoplasia is the major cause of cancer-related death in women worldwide. The carcinogenesis of cervical cancer is associated with excessive inflammation mediated by ROS. Tissue concentrations of H_2O_2 during inflammation can reach millimolar levels, while small amounts of H_2O_2 produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase have been suggested to affect the microenvironments of the plasma membrane (12,13). H_2O_2 affects essential functions, including cell growth, proliferation and differentiation, by altering signaling cascades and gene expression. H_2O_2 might also exert severe effects such as cell apoptosis and necrosis. The effects of H_2O_2 on the activities of MAPKs differ depending on the cell type and the experimental conditions, resulting in various cell responses. Exogenous H_2O_2 is often utilized as the representative ROS for regulating oxidative stress in cells. H_2O_2-induced cell death in cervical cancer cells may be toxicologically attractive in relation to the intracellular ROS and MAPKs.

Thus, in the present study, the effects of exogenous H_2O_2 on the cell growth and death of human cervical adenocarcinoma HeLa cells were investigated. The effects of various MAPK inhibitors, including *N*-acetyl cysteine (NAC) and propyl gallate (PG) (well-known antioxidants), and L-buthionine sulfoximine (BSO; an inhibitor of GSH synthesis), were also evaluated in H_2O_2-treated HeLa cells with respect to cell growth and death, as well as ROS and GSH levels.

Materials and methods

Cell culture. Human cervical adenocarcinoma HeLa cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in a humidified incubator containing 5% CO_2 at 37˚C. The cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco-BRL, Grand Island, NY, USA). The cells were then routinely grown in 100-mm plastic tissue culture dishes (Nunc A/S, Roskilde, Denmark) and harvested with a solution of trypsin-EDTA while in a logarithmic phase of growth.

Reagents. H_2O_2 was purchased from Sigma-Aldrich. JNK (SP600125), MEK (PD98059) and p38 inhibitors (SB203580) were purchased from Calbiochem (San Diego, CA, USA). The inhibitors were dissolved in Dulbecco's modified Eagle's medium (DMSO) at 10 mM as a stock solution. NAC, PG and BSO were obtained from Sigma-Aldrich. NAC was dissolved in 20 mM HEPES buffer (pH 7.0), PG was dissolved in ethanol at 200 mM as a stock solution and BSO was dissolved in water. Based on previous studies (8,14), the cells were pretreated with 10 μM of each MAPK inhibitor, 2 mM NAC, 100 μM PG or 10 μM BSO for 1 h prior to treatment with H_2O_2. Ethanol (0.2%) and DMSO (0.2%) were used as a control vehicle and they did not affect cell growth or death.

Cell growth assays. Cell growth changes were determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye (MTT; Sigma-Aldrich) absorbance in living cells as previously described (15). Briefly, $4x10^4$ cells/well were seeded in 96-well microtiter plates (Nunc A/S). Following exposure to 100 μM H_2O_2 for 24 h in the presence or absence of 10 μM of each MAPK inhibitor (2 mM NAC, 100 μM PG or 10 μM BSO) MTT solution [20 μl: 2 mg/ml in phosphate-buffered saline (PBS)] was added to each well of the 96-well plates. The plates were incubated for 4 h at 37˚C. Medium was withdrawn from the plates by pipetting and 200 μl DMSO was added to each well to solubilize the formazan crystals. Optical density was measured at 570 nm using a microplate reader (Synergy™ 2; BioTek Instruments Inc., Winooski, VT, USA).

Cell cycle and sub-G1 analysis. Cell cycle and sub-G1 analysis were determined by propidium iodide (PI, Ex/Em=488/617 nm; Sigma-Aldrich) staining as previously described (16). Briefly, $1x10^6$ cells in 60-mm culture dishes (Nunc A/S) were incubated with 100 μM H_2O_2 for 24 h in the presence or absence of 10 μM of each MAPK inhibitor, 2 mM NAC, 100 μM PG or 10 μM BSO. Total cells, including floating cells, were then washed with PBS and fixed in 70% (v/v) ethanol. The cells were washed again with PBS, and then incubated with PI (10 μg/ml) with simultaneous RNase treatment at 37˚C for 30 min. Cellular DNA content was measured using a FACStar flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and analyzed using Lysis II and CellFit software (Becton-Dickinson).

Annexin V-fluorescein isothiocyanate (FITC) staining for cell death detection. Apoptotic cell death was determined by staining the cells with Annexin V-FITC (Ex/Em=488/519 nm; Invitrogen Life Technologies, Camarillo, CA, USA) as previously described (17). Briefly, $1x10^6$ cells in 60-mm culture dishes were incubated with 100 μM H_2O_2 for 24 h in the presence or absence of 10 μM of each MAPK inhibitor, 2 mM NAC, 100 μM PG or 10 μM BSO. The cells were washed twice with cold PBS and then resuspended in 500 μl binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of $1x10^6$ cells/ml. Annexin V-FITC (5 μl) was then added, and the cells were analyzed with a FACStar flow cytometer.

Measurement of the mitochondrial membrane potential (MMP; $\Delta\Psi_m$). MMP ($\Delta\Psi_m$) levels were measured using a rhodamine 123 fluorescent dye (Sigma-Aldrich; Ex/Em=485/535 nm) as described previously (17,18). Briefly, $1x10^6$ cells in 60-mm culture dishes were incubated with 100 μM H_2O_2 for 24 h in the presence or absence of 10 μM of each MAPK inhibitor, 2 mM NAC, 100 μM PG or 10 μM BSO. The cells were washed twice with PBS and incubated with rhodamine 123 (0.1 μg/ml) at 37˚C for 30 min. Rhodamine 123 staining intensity was determined using a FACStar flow cytometer. Rhodamine 123-negative cells were characterized by loss of MMP ($\Delta\Psi_m$).

Detection of the intracellular ROS levels. Intracellular ROS levels were detected using an oxidation-sensitive fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Ex/Em=495/529 nm; Invitrogen Life Technologies)

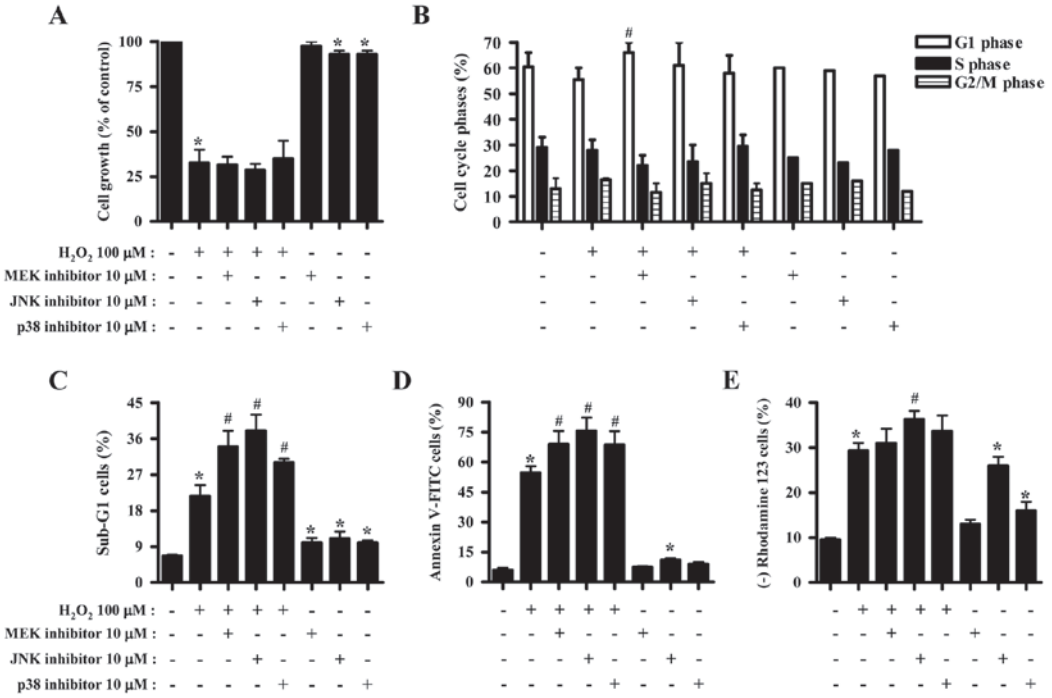

Figure 1. Effects of MAPK inhibitors on cell growth and death of H_2O_2-treated HeLa cells. Exponentially-growing cells were treated with 100 μM H_2O_2 for 24 h following a 1-h pre-incubation with each MAPK inhibitor (10 μM). The graphs show (A) HeLa cell growth changes as assessed by MTT assays, (B) HeLa cell cycle distribution as measured using a FACStar flow cytometer, (C) the percentages of sub-G1 cells as measured using a FACStar flow cytometer, (D) the percentages of Annexin V-FITC-positive cells as measured using a FACStar flow cytometer and (E) the percentages of a rhodamine 123-negative [MMP ($\Delta\Psi_m$) loss] cells as measured using a FACStar flow cytometer. *P<0.05 compared with the control cells; #P<0.05 compared with cells treated with H_2O_2 only.

and dihydroethidium (DHE, Ex/Em=518/605 nm; Invitrogen Life Technologies) as previously described (17,19). DHE is highly selective for $O_2^{\bullet-}$ among ROS. Briefly, 1x10^6 cells/ml in FACS tube (Becton-Dickinson) were treated with 100 μM H_2O_2 with or without 10 μM of each MAPK inhibitor in the presence of 20 μM H_2DCFDA or DHE. The levels of DCF and DHE fluorescence dyes were evaluated using a FACStar flow cytometer at 1 h of treatment. DCF (ROS) and DHE ($O_2^{\bullet-}$) levels were expressed as mean fluorescence intensity (MFI), which was calculated using CellQuest software (Becton-Dickinson). Moreover, 1x10^6 cells in 60-mm culture dishes (Nunc A/S) were incubated with 100 μM H_2O_2 for 24 h in the presence or absence of 10 μM of each MAPK inhibitor, 2 mM NAC, 100 μM PG or 10 μM BSO. The cells were then incubated with 20 μM H_2DCFDA or DHE at 37˚C for 30 min. H_2DCFDA or DHE fluorescence was assessed using a FACStar flow cytometer.

Detection of the intracellular GSH. Cellular GSH levels were analyzed using a 5-chloromethylfluorescein diacetate dye (CMFDA, Ex/Em=522/595 nm; Invitrogen Life Technologies) as previously described (19,20). Briefly, 1x10^6 cells/ml in FACS tube were treated with 100 μM H_2O_2 with or without 10 μM of each MAPK inhibitor in the presence of 5 μM CMFDA. The levels of CMF fluorescence were evaluated using a FACStar flow cytometer at 1 h of treatment. CMF (GSH) levels were expressed as MFI, which was calculated using CellQuest software. In addition, 1x10^6 cells in 60-mm culture dishes (Nunc A/S) were incubated with 100 μM H_2O_2 for 24 h in the presence or absence of 10 μM of each MAPK inhibitor, 2 mM NAC, 100 μM PG or 10 μM BSO. The cells were then incubated with 5 μM CMFDA at 37˚C for 30 min. CMF fluorescence was assessed using a FACStar flow cytometer. Negative CMF staining (GSH-depletion) of cells is expressed as the percentage of (-) CMF cells.

Statistical analysis. Results were the mean of at least two independent experiments (mean ± SD). Data were analyzed using GraphPad Prism4 software (GraphPad Prism, Inc., San Diego, CA, USA). Student's t-test or one-way analysis of variance with post hoc analysis using Tukey's multiple comparison test was used for parametric data. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of MAPK inhibitors on cell growth and death of H_2O_2-treated HeLa cells. The effect of H_2O_2 on the growth inhibitions of HeLa cells was examined using MTT assays. A concentration of 100 μM H_2O_2 was considered sufficient to differentiate the levels of cell growth and death in the presence or absence of each MAPK inhibitor. Exposure to 100 μM H_2O_2 for 24 h inhibited the growth of HeLa cells by ~70% (Fig. 1A). None of the MAPK inhibitors significantly affected the growth inhibition induced by H_2O_2 (Fig. 1A). JNK and p38 inhibitors slightly reduced the growth of HeLa control cells (Fig. 1A). Moreover, H_2O_2 did not significantly affect HeLa cell cycle distribution (Fig. 1B). While JNK and p38 inhibitors did not affect the cell cycle distribution of H_2O_2-treated HeLa cells, MEK inhibitor was found to increase the number of HeLa cells in the G1 phase of the cell cycle (Fig. 1B). H_2O_2 increased the number of HeLa cells in the sub-G1 phase by ~15% compared with H_2O_2-untreated HeLa control cells (Fig. 1C). All the

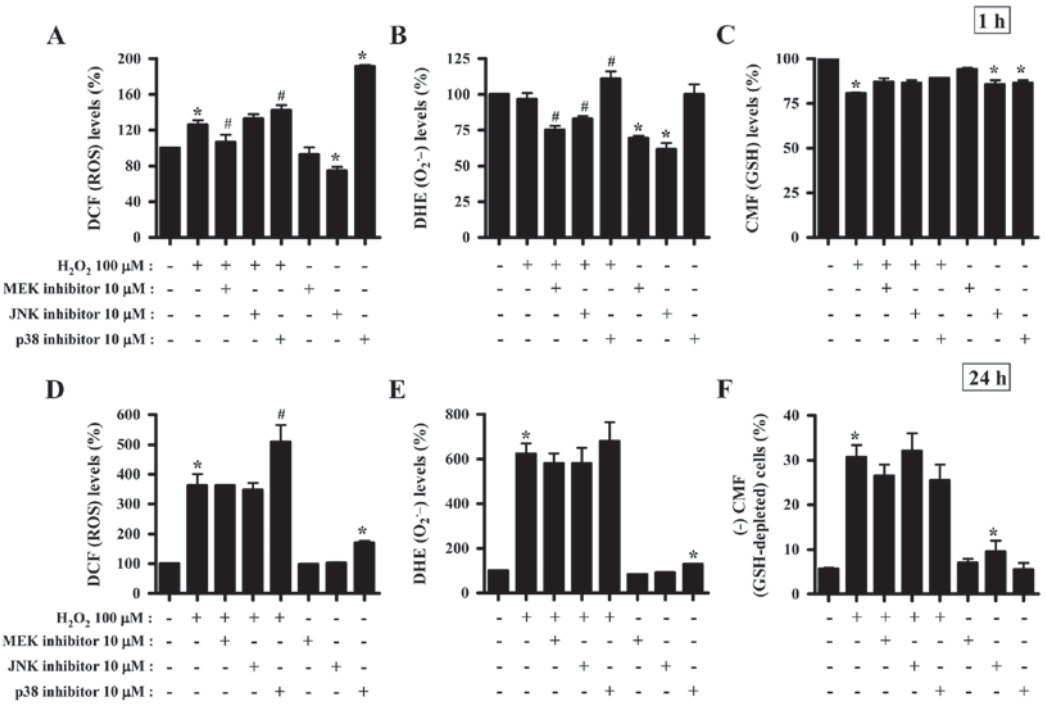

Figure 2. Effects of MAPK inhibitors on the intracellular reactive oxygen species (ROS) and glutathione (GSH) levels in H_2O_2-treated HeLa cells. Exponentially-growing cells were treated with 100 μM H_2O_2 for 1 or 24 h following a 1-h pre-incubation with each MAPK inhibitor (10 μM). ROS and GSH levels in HeLa cells were measured using a FACStar flow cytometer. The graphs indicate DCF (ROS) levels (%) at (A) 1 and (D) 24 h; DHE ($O_2^{\bullet-}$) levels (%) at (B) 1 and (E) 24 h; mean CMF (GSH) levels (%) at (C) 1 h and (F) (-) CMF (GSH-depleted) cells (%) at 24 h of treatment with H_2O_2 in HeLa cells compared with the control cells. *P<0.05 compared with the control cells; #P<0.05 compared with cells treated with H_2O_2 only.

MAPK inhibitors increased the number of H_2O_2-treated and control HeLa cells in the sub-G1 phase of the cell cycle (Fig. 1C). H_2O_2 increased the number of Annexin V-positive HeLa cells by ~50%, indirectly indicating that HeLa cell death induced by H_2O_2 occurred via apoptosis (Fig. 1D). MAPK inhibitors were found to significantly increase the number of Annexin V-FITC-positive H_2O_2-treated HeLa cells (Fig. 1D). Particularly, JNK inhibitor was found to exert a strong effect on cell death (Fig. 1C and D). JNK inhibitor alone increased the number of Annexin V-FITC-positive HeLa control cells (Fig. 1D). Cell death has been closely associated with the collapse of MMP ($\Delta\Psi_m$) (21). As expected, loss of MMP ($\Delta\Psi_m$) was observed in H_2O_2-treated HeLa cells (Fig. 1E). However, the percentage of cells with MMP ($\Delta\Psi_m$) loss was lower compared with that of Annexin V-FITC-positive cells. All the MAPK inhibitors slightly enhanced the loss of MMP ($\Delta\Psi_m$) in H_2O_2-treated HeLa cells, and JNK inhibitor exerted the most significant effect (Fig. 1E). JNK or p38 inhibitor alone triggered MMP ($\Delta\Psi_m$) loss in HeLa control cells (Fig. 1E).

Effects of MAPK inhibitors on the intracellular ROS and GSH levels in H_2O_2-treated HeLa cells. To determine whether the levels of intracellular ROS and GSH in H_2O_2-treated HeLa cells were changed by treatment with each MAPK inhibitor, ROS and GSH levels in HeLa cells were assessed at 1 and 24 h of H_2O_2 treatment (Fig. 2). Intracellular ROS (DCF) levels were increased in H_2O_2-treated cells at 1 (Fig. 1A) and 24 h (Fig. 2D). The MEK inhibitor appeared to attenuate the increased ROS (DCF) levels induced by H_2O_2 treatment for 1 h (Fig. 2A). The p38 inhibitor enhanced the increased ROS (DCF) levels induced by H_2O_2 treatment for 1 (Fig. 2A) and 24 h (Fig. 2D). The JNK inhibitor was found to significantly decrease ROS levels in HeLa control cells at 1 h, while the p38 inhibitor increased ROS levels in these cells at 1 (Fig. 2A) and 24 h (Fig. 2D). Moreover, red fluorescence derived from DHE reflecting the intracellular $O_2^{\bullet-}$ levels was not altered in H_2O_2-treated HeLa cells at 1 h (Fig. 2B), while it was significantly increased at 24 h of H_2O_2 treatment (Fig. 2E). The MEK and JNK inhibitors decreased DHE ($O_2^{\bullet-}$) levels in H_2O_2-treated and -untreated HeLa cells at 1 h, while the p38 inhibitor increased the DHE ($O_2^{\bullet-}$) levels in H_2O_2-treated HeLa cells (Fig. 2B). At 24 h of H_2O_2 treatment, none of the MAPK inhibitors significantly changed DHE ($O_2^{\bullet-}$) levels in H_2O_2-treated HeLa cells, and p38 inhibitor alone increased DHE ($O_2^{\bullet-}$) levels in HeLa control cells (Fig. 2E). H_2O_2 decreased GSH levels in HeLa cells at 1 h of treatment (Fig. 2C) as measured using a CMF fluorescence dye. All the MAPK inhibitors were shown to attenuate the decreased GSH levels induced by H_2O_2 treatment for 1 h and to decrease the basal levels of GSH in HeLa control cells (Fig. 2C). H_2O_2 increased the percentages of GSH-depleted HeLa cells at 24 h of treatment by ~25% compared with the H_2O_2-untreated HeLa control cells (Fig. 2F). MEK and p38 inhibitors were found to attenuate the depletion of GSH in H_2O_2-treated HeLa cells, and JNK inhibitor alone induced the depletion of GSH in HeLa control cells (Fig. 2F).

Effects of NAC, PG and BSO on cell growth and death of H_2O_2-treated HeLa cells. The effects of NAC, PG or BSO on cell growth, cell death and MMP ($\Delta\Psi_m$) in H_2O_2-treated HeLa cells were assessed at 24 h of treatment. NAC and PG significantly attenuated the growth inhibition induced by

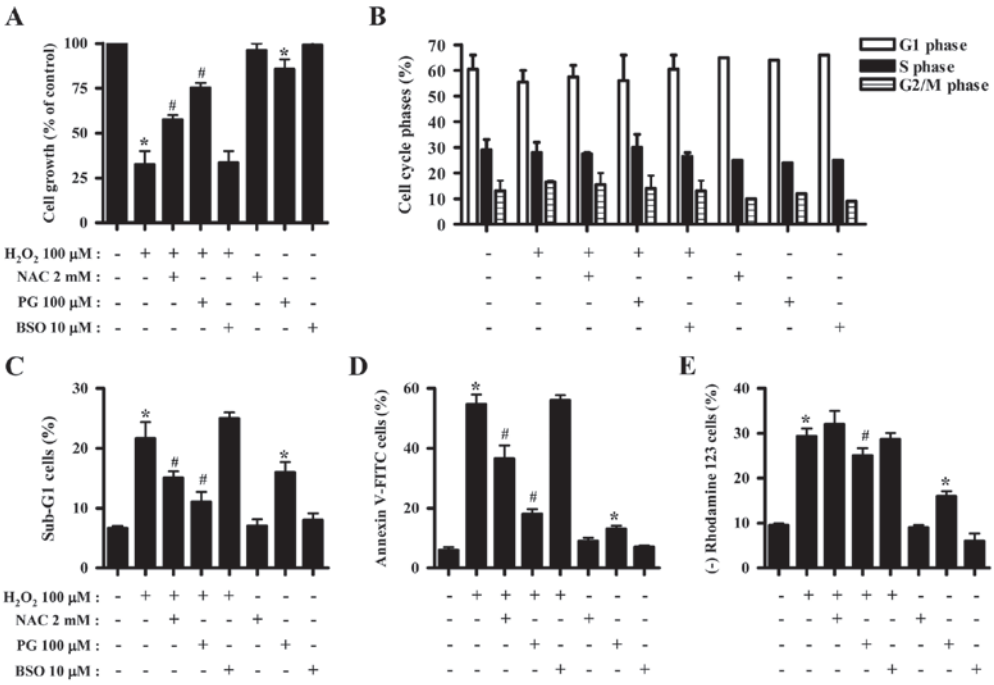

Figure 3. Effects of *N*-acetyl cysteine (NAC), propyl gallate (PG) and L-buthionine sulfoximine (BSO) on cell growth and death of H_2O_2-treated HeLa cells. Exponentially-growing cells were treated with 100 μM H_2O_2 for 24 h following a 1-h pre-incubation with 2 mM NAC, 100 μM PG or 10 μM BSO. The graphs show (A) HeLa cell growth changes, (B) HeLa cell cycle distribution, (C) the percentages of sub-G1 cells, (D) the percentages of Annexin V-FITC-positive cells and (E) the percentages of a rhodamine 123-negative [MMP ($\Delta\Psi_m$) loss] cells. *P<0.05 compared with the control cells; #P<0.05 compared with cells treated with H_2O_2 only.

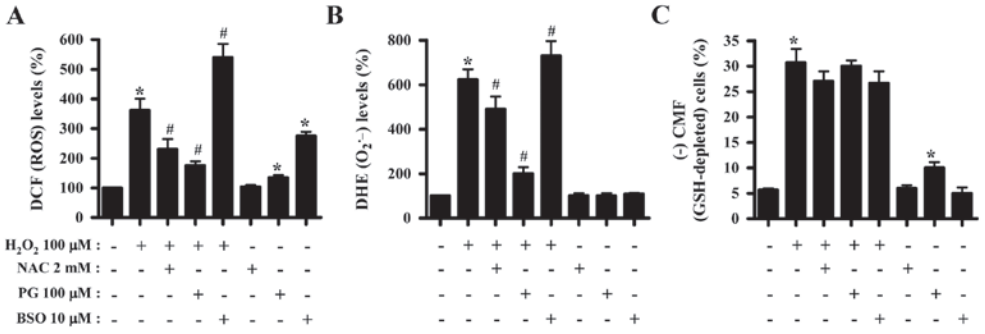

Figure 4. Effects of *N*-acetyl cysteine (NAC), propyl gallate (PG) and L-buthionine sulfoximine (BSO) on the intracellular reactive oxygen species (ROS) and glutathione (GSH) levels in H_2O_2-treated HeLa cells. Exponentially-growing cells were treated with 100 μM H_2O_2 for 24 h following a 1-h pre-incubation with 2 mM NAC, 100 μM PG or 10 μM BSO. ROS and GSH levels in HeLa cells were measured using FACStar flow cytometry. The graphs indicate (A) DCF (ROS) levels (%), (B) DHE ($O_2^{\bullet-}$) levels (%) and (C) (-) CMF (GSH-depleted) cells (%) compared with the control cells. *P<0.05 compared with the control cells. #P<0.05 compared with cells treated with H_2O_2 only.

H_2O_2, where PG exerted a stronger effect (Fig. 3A). However, BSO did not affect cell growth of the H_2O_2-treated HeLa cells (Fig. 3A). Only PG reduced the cell growth in HeLa control cells (Fig. 3A). Concerning cell cycle analysis, NAC, PG or BSO did not alter the cell cycle distribution of H_2O_2-treated HeLa cells (Fig. 3B). NAC and PG decreased the percentage of H_2O_2-treated HeLa cells in the sub-G1 phase, while BSO slightly increased the percentage in sub-G1 cells (Fig. 3C). Notably, PG significantly increased the percentage of sub-G1 HeLa control cells (Fig. 3C). Moreover, NAC and PG significantly reduced the percentage of Annexin V-FITC-positive H_2O_2-treated HeLa cells, and PG markedly prevented HeLa cell death induced by H_2O_2 (Fig. 3D). In addition, PG alone increased the percentage of Annexin V-FITC-positive HeLa control cells (Fig. 3D). With respect to MMP ($\Delta\Psi_m$), PG decreased the loss of MMP ($\Delta\Psi_m$) induced by H_2O_2 to some extent, while NAC and BSO did not significantly affect the loss of MMP ($\Delta\Psi_m$) (Fig. 3E). PG also increased the percentage of HeLa control cells with MMP ($\Delta\Psi_m$) loss (Fig. 3E).

Effects of NAC, PG and BSO on intracellular ROS and GSH levels in H_2O_2-treated HeLa cells. Whether the levels of intracellular ROS and GSH in H_2O_2-treated HeLa cells were changed by treatment with NAC, PG or BSO was subsequently investugated. Both NAC and PG suppressed the increased ROS levels in H_2O_2-treated HeLa cells, while BSO enhanced the increased ROS levels induced by H_2O_2 (Fig. 4A). Additionally, PG and BSO significantly increased ROS (DCF)

levels in HeLa control cells (Fig. 4A). Similarly, NAC and PG decreased the augmented $O_2^{\bullet-}$ levels in H_2O_2-treated HeLa cells, in contrast to BSO which increased $O_2^{\bullet-}$ levels (Fig. 4B). Concerning assessment of the GSH levels, NAC appeared to reduce the percentage of GSH-depleted H_2O_2-treated HeLa cells, while PG did not affect the depletion of GSH (Fig. 4C). PG alone significantly induced the depletion of GSH in HeLa control cells (Fig. 4C). Notably, treatment with 10 μM BSO failed to enhance the depletion of GSH in H_2O_2-treated HeLa cells, which instead slightly attenuated GSH depletion in these cells (Fig. 4C).

Discussion

Since H_2O_2 inhibited HeLa cell growth and induced HeLa cell death, the present study aimed to evaluate the toxicological effect of H_2O_2 on the cell growth and death of HeLa cells following treatment with MAPK inhibitors, NAC, PG or BSO. H_2O_2 increased the number of Annexin V-FITC-positive HeLa cells. The activity of caspase-3 was also found to be increased in H_2O_2-treated HeLa cells (data not shown), indicating that the H_2O_2-induced HeLa cell death occurred via apoptosis. In addition, H_2O_2 triggered the loss of MMP ($\Delta\Psi_m$) in HeLa cells, suggesting that cell death by H_2O_2 was correlated with the collapse of MMP ($\Delta\Psi_m$). H_2O_2 did not induce any specific phase arrests of the HeLa cell cycle, indicating that the H_2O_2-induced oxidative stress did not affect particular proteins related to cell cycle arrest and progression.

ERK activation was observed in H_2O_2-treated HeLa cells (22). In the present study, the MEK inhibitor, which presumably inactivates ERK, did not affect the H_2O_2-induced inhibition of HeLa cell growth, while it increased cell death. In addition, the MEK inhibitor alone increased the number of HeLa control cells in the sub-G1 phase of the cell cycle. Thus, ERK is likely to be associated with cell survival rather than cell death and proliferation. JNK and p38 MAPKs are known to be related to cell death (6,7). H_2O_2 has been shown to increase the activity of JNK and p38 in HeLa cells (22,23). Yamagishi *et al* (23) demonstrated that HeLa cell apoptosis induced by 500 μM H_2O_2 was suppressed by treatment with p38 inhibitor but not JNK inhibitor, suggesting that apoptosis occurs through a p38 MAPK-dependent signaling pathway. However, the results of the present study indicate that treatment with JNK and p38 inhibitors significantly increased the cell death of HeLa cells treated with 100 μM H_2O_2. These results suggest that JNK and p38 signaling pathways in HeLa cells treated with 100 μM H_2O_2 are involved in a pro-survival function. The difference potentially resulted from the different concentrations and incubation times used in each experiment since the effects of MAPKs are altered by the different types of oxidative stress. JNK and p38 inhibitors significantly induced cell growth inhibition, cell death and MMP ($\Delta\Psi_m$) loss in HeLa control cells, indicating that the basal activities of these MAPKs are involved in the cell growth and survival of HeLa cells. Regarding the loss of MMP ($\Delta\Psi_m$), none of the MAPK inhibitors markedly enhanced the loss of MMP ($\Delta\Psi_m$) in H_2O_2-treated HeLa cells compared with HeLa cell death. Thus, the loss of MMP ($\Delta\Psi_m$) was not likely to correlate with apoptosis in HeLa cells treated with H_2O_2 and each MAPK inhibitor. Moreover, JNK and p38 inhibitors did not change the cell cycle distributions in H_2O_2-treated HeLa cells, while the MEK inhibitor increased the number of cells in the G1 phase of the cell cycle. The underlying mechanisms of the cell cycle regulation by MAPKs should be further investigated under oxidative stress.

ROS levels, including $O_2^{\bullet-}$, were significantly increased in HeLa cells treated with H_2O_2 for 24 h. It is suggested that exogenous H_2O_2 strongly generates $O_2^{\bullet-}$ by damaging the mitochondria, and both H_2O_2 and $O_2^{\bullet-}$ can be efficiently converted into the toxic $^{\bullet}OH$ via the Fenton reaction to kill HeLa cells. However, H_2O_2 did not increase $O_2^{\bullet-}$ (DHE) levels in HeLa cells at 1 h of treatment, suggesting that it does not affect the mitochondrial respiratory transport chain and the activity of various oxidases to generate $O_2^{\bullet-}$. MEK inhibitor showing a proapoptotic effect on H_2O_2-treated HeLa cells did not alter ROS levels, including $O_2^{\bullet-}$, at 24 h of treatment, while it decreased ROS levels in H_2O_2-treated and -untreated HeLa cells at 1 h of treatment. MEK inhibitor appeared to act as an antioxidant at 1 h of treatment, while it did not suppress HeLa cell death at 24 h of treatment. The JNK inhibitor also did not alter the ROS levels in H_2O_2-treated and -untreated HeLa cells at 24 h. Instead, this inhibitor reduced $O_2^{\bullet-}$ levels in H_2O_2-treated HeLa cells at 1 h of treatment and it also decreased basal ROS levels in HeLa control cells. Thus, ERK and JNK signaling pathways in H_2O_2-treated and -untreated HeLa cells did not significantly influence redox state to affect HeLa cell death. The p38 inhibitor enhanced ROS levels in H_2O_2-treated and -untreated HeLa cells at 1 and 24 h of treatment, suggesting that p38 signaling is involved in cell survival and the antioxidant system in HeLa cells. Since changes in ROS levels regulated by each MAPK inhibitor and outcomes of these signaling by different types of oxidant stress are complex in cells, the diverse functions of each MAPK inhibitor under the different oxidative states were then investigated with regard to cell survival or death. NAC, a well-known antioxidant, attenuated the growth inhibition and cell death of H_2O_2-treated HeLa cells. As expected, NAC markedly decreased ROS levels, including $O_2^{\bullet-}$, in H_2O_2-treated HeLa cells. BSO increased ROS levels in H_2O_2-treated and -untreated HeLa cells. However, growth inhibition and cell death were not affected. The BSO-increased ROS levels may not be sufficient to increase cell death in these cells.

PG is known to be a synthetic antioxidant (24,25), while it has been suggested to possess prooxidant properties (26-28). Antioxidant and cytoprotective properties of PG may change to prooxidant, cytotoxic and genotoxic in the presence of Cu(II) (29). According to a previous study, ROS levels, including $O_2^{\bullet-}$, were demonstrated to be increased or decreased in PG-treated HeLa cells depending on the incubation times and doses (19). The results of the present study indicate that PG alone slightly inhibited the growth of HeLa cells and induced cell death accompanied by the loss of MMP ($\Delta\Psi_m$). In addition, PG slightly increased ROS levels in HeLa cells at 24 h and it also increased $O_2^{\bullet-}$ (DHE) levels at 1 h of treatment (data not shown). Thus, it is conceivable that PG generates $O_2^{\bullet-}$ in HeLa cells by impairing the mitochondrial function, consequently leading to HeLa cell death via oxidative stress. Notably, PG significantly attenuated growth inhibition and cell death in H_2O_2-treated HeLa cells. Moreover, PG markedly reduced the increased ROS levels, including $O_2^{\bullet-}$, by H_2O_2 treatment. Therefore, PG was found to protect HeLa cells against

exogenous H_2O_2 by reducing H_2O_2-induced oxidative stress. Thus, PG acts as an antioxidant (24,25) or prooxidant (26-28) depending on various conditions, such as incubation times and doses, co-incubation drugs and cell types. In addition, PG did not strongly attenuate the loss of MMP ($\Delta\Psi_m$) following H_2O_2 treatment. Moreover, NAC failed to prevent the loss of MMP ($\Delta\Psi_m$) by H_2O_2 treatment. Since the levels of MMP ($\Delta\Psi_m$) loss in H_2O_2-treated HeLa cells was relatively low compared with that of Annexin V-FITC-positive cells, the loss of MMP ($\Delta\Psi_m$) by H_2O_2 was expected to be essential but not sufficient to induce apoptosis in HeLa cells. Regarding cell cycle changes, none of the NAC, PG or BSO significantly altered the cell cycle distributions in H_2O_2-treated HeLa cells, suggesting that changes in ROS levels did not specifically regulate the cell cycle to induce particular phase arrests in HeLa cells.

Apoptotic effects are inversely proportional to GSH content (20,30,31). Similarly, H_2O_2 increased the percentages of GSH-depleted HeLa cells at 24 h of treatment. The JNK inhibitor and PG also significantly induced the depletion of GSH in HeLa control cells. These results support the hypothesis that the intracellular GSH content has a decisive impact on cell death (18,20,32). However, none of the MAPK inhibitors enhanced the depletion of GSH in H_2O_2-treated HeLa cells, while NAC failed to prevent the depletion of GSH. Furthermore, PG partially recovered the depletion of GSH in H_2O_2-treated HeLa cells. Therefore, the loss of GSH content appeared to be necessary, but not sufficient to induce apoptosis in H_2O_2-treated HeLa cells. H_2O_2 decreased GSH levels at 1 h of treatment. The decreased GSH levels were likely to decrease in order to reduce ROS (DCF) levels. In addition, MAPK inhibitors partially recovered GSH levels in H_2O_2-treated HeLa cells and reduced basal GSH levels in HeLa control cells. Thus, these results suggest that MAPK inhibitors differentially regulate the intracellular GSH levels in HeLa cells depending on the presence or absence of H_2O_2. Notably, treatment with 10 μM BSO showing an increased effect on ROS levels did not intensify the depletion of GSH in H_2O_2-treated HeLa cells. Previous studies have demonstrated that 1 or 10 μM BSO significantly enhanced the depletion of GSH in arsenic trioxide-treated HeLa (30) and A549 cells (33). Additional studies have shown that >100 μM BSO decreased GSH levels in breast cancer (34) and leukemia cells (35). Therefore, these results suggest that BSO differentially affects GSH levels depending on the concentration used, the cell type and co-incubation drugs.

In conclusion, H_2O_2 induced growth inhibition and death in HeLa cells, which was accompanied by intracellular increase in ROS levels and GSH depletion. MAPK inhibitors generally enhanced H_2O_2-induced HeLa cell death. Particularly, the p38 inhibitor increased ROS levels in H_2O_2-treated HeLa cells. NAC and PG attenuated H_2O_2-induced HeLa cell growth inhibition and death together with the suppression of ROS levels. The present study provides insight into the toxicological effects of exogenous H_2O_2 on HeLa cells with respect to MAPK signaling and antioxidants.

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