Anti-apoptotic effect of caspase inhibitors on H₂O₂-treated HeLa cells through early suppression of its oxidative stress

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Abstract. Oxidative stress-induced cytotoxicity in cervical cancer cells may be of toxicological interest. In the present study, the effects of exogenous H₂O₂ on cell growth and death in HeLa cervical cancer cells were investigated, and the anti-apoptotic effects of various caspase (pan-caspase, caspase-3, -8 or -9) inhibitors on H₂O₂-treated HeLa cells were also evaluated with regard to reactive oxygen species (ROS) and glutathione (GSH) levels. Based on MTT assays, H₂O₂ inhibited the growth of HeLa cells with an IC₅₀ value of ~75 μM at 24 h. H₂O₂ increased the number of dead cells and Annexin V-FITC-positive cells in the HeLa cells, which was accompanied by the activation of caspase-3 and the loss of mitochondrial membrane potential (ΔΨₘ). However, relatively higher doses of H₂O₂ induced necrosis in HeLa cells. Caspase inhibitors significantly prevented H₂O₂-induced HeLa cell death. H₂O₂ increased ROS including O₂⁻ at 24 h and increased the activity of catalase in HeLa cells. H₂O₂ also increased the ROS level at 1 h, and several caspase inhibitors attenuated the increased level at 1 h but not at 6, 12 and 24 h. H₂O₂ decreased the GSH level in HeLa cells at 1 h, and several caspase inhibitors attenuated the decreased level of GSH at this time. H₂O₂ induced GSH depletion at 24 h. In conclusion, H₂O₂ inhibited the growth of HeLa cells via apoptosis and/or necrosis, which was accompanied by intracellular increases in ROS levels and GSH depletion. Caspase inhibitors are suggested to suppress H₂O₂-induced oxidative stress to rescue HeLa cells at the early time point of 1 h.

Introduction

Reactive oxygen species (ROS) are a group of oxygen moieties, which include hydrogen peroxide (H₂O₂), the superoxide anion (O₂⁻) and the hydroxyl radical (•OH). Conventional theory has regarded ROS as deleterious or harmful to cells (1). However, it has become clear that ROS delicately regulate many cellular functions such as gene expression, differentiation and cell proliferation (2). They can also act as second messengers, influencing discrete signal transduction pathways in a variety of systems (3, 4). ROS are continuously generated by the respiratory chain during oxidative phosphorylation in the form of the O₂⁻• and/or are specifically produced by oxidases such as nicotine adenine diphosphate oxidase and xanthine oxidase (5). O₂⁻• is metabolized to H₂O₂ by superoxide dismutases (SODs) (6). Moreover, H₂O₂ by catalase or glutathione (GSH) peroxidase yields O₂ and H₂O (7). Since a change in the redox state of a tissue implies an alteration in ROS generation or metabolism, cellular ROS are tightly regulated to prevent tissue damage. Oxidative stress may be the consequence of either overproduction of ROS and/or downregulation of antioxidants; this stress is believed to be responsible for a variety of pathological conditions such as inflammation, cardiovascular disease and cancer (8-11).

Compared with other members of ROS, H₂O₂ plays a pivotal role since it is able to freely travel through biological membranes to a distance of several cell diameters and interacts with ferrous iron (Fenton chemistry) causing the formation of the very aggressive and short-lived •OH. Tissue concentrations of H₂O₂ for the period of inflammation have been likely to reach close to millimolar levels whereas tiny amounts of H₂O₂ generated by NADPH oxidase are assumed to take action only in microenvironments of the plasma membrane such as lipid rafts (12, 13). Nevertheless, in both cases, H₂O₂ may amend essential cellular functions of cell growth, proliferation and differentiation via altering signaling cascades and gene expression, or its higher level may lead to outcomes such as apoptosis or necrosis. Exogenous H₂O₂ is often applied as the
representative ROS in modeling oxidative stress in the cell and tissue.

The mechanism of apoptosis generally involves two signaling pathways, the mitochondrial pathway and the cell death receptor pathway (14-16). The key constituent in the mitochondrial pathway is the efflux of cytochrome c from mitochondria to the cytosol, where it subsequently forms a complex (apoptosome) with Apaf-1 and caspase-9, activating other caspases including caspase-3 and -7 (17). The cell death receptor pathway is characterized by binding cell death ligands such as TNFα and Fas and their cell death receptors, and subsequently activates caspase-8 and -3 (18,19). Particularly, cytosolic BID is cleaved by caspase-8 to generate a truncated product (tBID), which translocates to the mitochondria and decreases mitochondrial membrane potential (MMP; ΔΨm) resulting in release of cytochrome c. Therefore, crosstalk between both apoptotic pathways is manifested by the tBID. Caspase-3 is an executioner caspase, whose activation can systematically dismantle cells by cleaving key proteins such as poly(ADP-ribose) polymerase (PARP).

Cervical cancer is a major cause of cancer-related death in women worldwide, and the occurrence of this cancer is ascribed to changes in cancer-related genes as well as environmental events including viral infections. The carcinogenesis of cervical cancer has been known to be tightly linked to tissue inflammation mediated by ROS. Moreover, ROS influence genetic and epigenetic changes thereby modulating cellular proliferation and differentiation (11). H2O2-induced cytotoxicity in cervical cancer cells may be of toxicological research interest. Thus, in the present study, the effects of exogenous H2O2 on cell growth and death in human cervix adenocarcinoma HeLa cells were investigated and the anti-apoptotic effects of various caspase (pan-caspase, caspase-3, -8 or -9) inhibitors on H2O2-treated HeLa cells were evaluated in relation to changes in 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% CO2 at 37°C. HeLa cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (both from Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and 1% penicillin-streptomycin (Gibco-BRL, Grand Island, NY, USA). Cells were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and harvested with a solution of trypsin-EDTA while in a logarithmic phase of growth.

Reagents. H2O2 was purchased from Sigma-Aldrich Chemical Co. The pan-caspase inhibitor (Z-VAL-FMK; benzoyloxy carbonyl-Val-Ala-Asp-fluoromethylketone), caspase-3 inhibitor (Z-DEVD-FMK; benzoyloxy carbonyl-Ala-Glu-Val-Asp-fluoromethylketone), caspase-8 inhibitor (Z-LETD-FMK; benzoyloxy carbonyl-Ile-Glu-Thr-Asp-fluoromethylketone) and caspase-9 inhibitor (Z-LEHD-FMK; benzoyloxy carbonyl-LeuGlu-His-Asp-fluoromethylketone) were obtained from R&D Systems, Inc. (Minneapolis, MN, USA) and were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich Chemical Co.).

Based on a previous study (20), cells were pretreated with each caspase inhibitor for 1 h prior to treatment with H2O2. DMSO (0.2%) was used as a control vehicle and it did not appear to affect cell growth or death.

Cell growth and cell number assays. Cell growth changes were determined by measuring the absorbance of 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide dye (MTT; Sigma-Aldrich Chemical Co.) in living cells as described previously (21). Changes in the numbers of viable and dead cells were determined by trypan blue cell counting. In brief, 5x105 cells/well were seeded in 96-well microtiter plates for the MTT assays and 3x105 cells/well were seeded in 24-well plates (both from Nunc) for cell counting. After exposure to the indicated amounts of H2O2 for 24 h, the cells in the 96-well plates were used for MTT assays, and the cells in the 24-well plates were collected with trypsin digestion for trypan blue cell counting. Twenty microliters of MTT solution (2 mg/ml in phosphate-buffered saline (PBS)) was added to each well of the 96-well plates. The plates were incubated for an additional 4 h at 37°C. Media in plates were withdrawn by pipetting, and 200 µl DMSO was added to each well to solubilize the formazan crystals. The optical density was measured at 570 nm using a microplate reader (Synergy™ 2; BioTek Instruments Inc., Winooski, VT, USA).

Analysis of cell cycle distribution and sub-G1 phase cells. Cell cycle distribution and sub-G1 cell analysis were determined by propidium iodide (PI) (Sigma-Aldrich; Ex/Em = 488/617 nm) staining. In brief, 1x106 cells in a 60-mm culture dish (Nunc) were incubated with the indicated amounts of H2O2 with or without 15 µM caspase inhibitors for 1, 6, 12 or 24 h. Total cells including floating cells were then washed with PBS and fixed in 70% (v/v) ethanol. 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 and analyzed using Lysis II and CellFit software (both from Becton-Dickinson, Franklin Lakes, NJ, USA).

Lactate dehydrogenase (LDH) activity for the detection of necrosis. Necrosis in cells treated with H2O2 was evaluated using the LDH kit (Sigma-Aldrich Chemical Co.). In brief, 1x105 cells in a 60-mm culture dish (Nunc) were incubated with the indicated doses of H2O2 for 24 h. After treatment, the cell culture media were collected and centrifuged for 5 min at 1,500 rpm. Fifty microliters of the media supernatant was added to a fresh 96-well plate along with the LDH assay reagent and then incubated at room temperature for 30 min. The absorbance values were measured at 490 nm using a microplate reader (Synergy™ 2). LDH release was expressed as the percentage of extracellular LDH activity compared with the control cells.

Annexin V-FITC/PI staining for cell death detection. Apoptotic cell death was determined by staining the cells with Annexin V-fluorescein isothiocyanate (FITC; Invitrogen Life Technologies, Camarillo, CA, USA; Ex/Em = 488/519 nm) as previously described (22). In brief, 1x106 cells in a 60-mm culture dish (Nunc) were incubated with the designated
doses of H$_2$O$_2$ with or without 15 µM caspase inhibitors for 1, 6, 12 or 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 1x10$^6$ cells/ml. Annexin V-FITC (5 µl) and PI (1 µg/ml) were then added, and the cells were analyzed with a FACStar flow cytometer. Viable cells were negative for both PI and Annexin V; apoptotic cells were positive for Annexin V and negative for PI whereas late apoptotic dead cells display both high Annexin V and PI labeling. Nonviable cells, which underwent necrosis, were positive for PI and negative for Annexin V.

**Measurement of mitochondrial membrane potential (MMP; \( \Delta \Psi _{m} \)).** MMP (\( \Delta \Psi _{m} \)) levels were measured by Rhodamine 123 fluorescent dye (Sigma-Aldrich Chemical Co.; Ex/Em = 485/535 nm). In brief, 1x10$^6$ cells in a 60-mm culture dish (Nunc) were incubated with the indicated amounts of H$_2$O$_2$ with or without 15 µM caspase inhibitors for 24 h. 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 by a FACStar flow cytometer (Becton-Dickinson). Rhodamine 123-negative cells indicated the loss of MMP (\( \Delta \Psi _{m} \)) in the cells.

**Western blot analysis.** The change in caspase-3 and PARP in H$_2$O$_2$-treated cells was determined by western blotting. In brief, 1x10$^6$ cells in a 60-mm culture dish (Nunc) were incubated with the indicated amounts of H$_2$O$_2$ for 24 h. The cells were then washed in PBS and suspended in five volumes of lysis buffer [20 mM HEPES, (pH 7.9), 20% (v/v) glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% (v/v) NP-40, 0.5 mM DTT and 1% (v/v) protease inhibitor cocktail]. The protein concentrations in the supernatant were determined using the Bradford method. Samples containing 10 µg total protein were resolved by 8 or 12.5% SDS-PAGE gels, transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA, USA) by electroblotting and then probed with anti-caspase-3, anti-PARP, anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-LC3A/B (Cell Signaling Technology, Waltham, MA, USA) antibodies. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Blots were developed using an ECL kit (Amersham, Arlington Heights, IL, USA).

**Quantification of caspase-3 and -8 activities.** The activities of caspase-3 and -8 were assessed using the Caspase-3 and Caspase-8 Colorimetric Assay Kits (R&D Systems, Inc.) as previously used (23). In brief, 1x10$^6$ cells in a 60-mm culture dish (Nunc) were incubated with 100 µM H$_2$O$_2$ for 24 h. The cells were then washed in PBS and suspended in 5 volumes of lysis buffer provided in the kits. Protein concentrations were determined using the Bradford method. Supernatant samples containing 50 µg total protein were used for determination of caspase-3 and -8 activities. These were added to each well in 96-well microtiter plates (Nunc) with DEVD-pNA or IETD-pNA as caspase-3 and -8 substrates respectively at 37°C for 1 h. The optical density of each well was measured at 405 nm using a microplate reader (SpectraMax 340; Molecular Devices Co. Sunnyvale, CA, USA). Caspase-3 and -8 activities were expressed in arbitrary absorbance units.

**Detection of intracellular ROS levels.** Intracellular ROS levels were detected by the fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) (Ex/Em = 495/529 nm; Invitrogen Molecular Probes, Eugene, OR, USA) at 1, 6, 12 or 24 h. H$_2$DCFDA is poorly selective for the superoxide anion radical (O$_2^\bullet$). In contrast, dihydroethidium (DHE) (Invitrogen Molecular Probes; Ex/Em = 518/605 nm) is a fluorescent probe that is highly selective for O$_2^\bullet$ among ROS. In brief, 1x10$^6$ cells/ml in FACS tube (Becton-Dickinson) were treated with 100 µM H$_2$O$_2$ with or without 15 µM caspase inhibitors in the presence of 20 µM H$_2$DCFDA or DHE. The fluorescence levels of DCF and DHE were evaluated using a FACStar flow cytometer at 1 h. DCF (ROS) and DHE (O$_2^\bullet$) levels were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software (Becton-Dickinson). In addition, 1x10$^6$ cells in a 60-mm culture dish (Nunc) were incubated with the indicated amounts of H$_2$O$_2$ with or without 15 µM caspase inhibitors for 6, 12 and 24 h. Cells were incubated with 20 µM H$_2$DCFDA or DHE at 37°C for 30 min. H$_2$DCFDA or DHE fluorescence was assessed using a FACStar flow cytometer.

**Measurement of cellular SOD and catalase activities.** SOD enzyme activity was measured using the SOD assay kit-WST (Fluka Co., Milwaukee, WI, USA), and catalase enzyme activity was measured using a catalase assay kit from Sigma-Aldrich Chemical Co. In brief, 1x10$^6$ cells were incubated with 100 µM H$_2$O$_2$ 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% (v/v) glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% (v/v) NP-40, 0.5 mM DTT and 1% (v/v) protease inhibitor cocktail (from Sigma)]. The protein concentration of the supernatant was determined by the Bradford method. Supernatant samples containing 100 µg total protein were used for determination of SOD and catalase enzyme activities. These were added to each well in 96-well microtiter plates (Nunc) with the appropriate working solutions (according to the manufacturer’s instructions) at 25°C for 30 min. The color changes were measured at 450 or 520 nm using a microplate reader (SpectraMax 340). The value for the experimental group was expressed as a percentage of the control group.

**Detection of the intracellular GSH.** Cellular GSH levels were analyzed using a 5-chloromethylfluorescein diacetate dye (CMFDA) (Invitrogen Molecular Probes; Ex/Em = 522/595 nm) at 1, 6, 12, or 24 h. In brief, 1x10$^6$ cells/ml in a FACS tube (Becton-Dickinson) were treated with 100 µM H$_2$O$_2$ with or without 15 µM caspase inhibitors in the presence of 5 µM CMFDA. The level of CMF fluorescence was evaluated using a FACStar flow cytometer at 1 h. CMF (GSH) levels were expressed as MFI, which were calculated by CellQuest software. In addition, 1x10$^6$ cells in a 60-mm culture dish (Nunc) were incubated with the indicated amounts of H$_2$O$_2$ with or without 15 µM caspase inhibitors for 6, 12 and 24 h. Cells were incubated with 5 µM CMFDA at 37°C for 30 min. CMF fluorescence was assessed using a FACStar flow cytometer.
Results

Effects of \( \text{H}_2\text{O}_2 \) on cell growth in HeLa cells. The effect of \( \text{H}_2\text{O}_2 \) on the growth of HeLa cells was examined at 24 h. Treatment with 50-250 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) significantly decreased the viable (trypan blue-negative) cell number in the HeLa cells in a dose-dependent manner whereas \( \text{H}_2\text{O}_2 \) dose-dependently increased the number of dead (trypan blue-positive) cells (Fig. 1A).

Statistical analysis. The results represent the means of at least two independent experiments (means ± SD). The data were analyzed using InStat software (GraphPad Prism4; GraphPad Software, San Diego, CA, USA). 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. The statistical significance was defined as \( \text{p}<0.05 \).

Effects of \( \text{H}_2\text{O}_2 \) on cell death and MMP (\( \Delta \text{Ψ}_m \)) in HeLa cells. Next, we aimed to ascertain whether the \( \text{H}_2\text{O}_2 \)-induced cell death was through apoptosis or necrosis in HeLa cells.
50 or 100 µM H$_2$O$_2$ significantly increased the percentages of sub-G1 cells in HeLa cells; 250 µM H$_2$O$_2$ did not increase the percentages of sub-G1 cells in these cells (Fig. 2A). Since H$_2$O$_2$ can induce necrosis in HeLa cells, the status of necrosis was assessed using the LDH release assay. Treatment with 100 or 250 µM H$_2$O$_2$ significantly induced LDH release in HeLa cells at 24 h (Fig. 2B). Treatment with 50-250 µM H$_2$O$_2$ increased the numbers of Annexin V-FITC-positive cells in the HeLa cells in a dose-dependent manner (Fig. 2C). Treatment with 100 µM H$_2$O$_2$ increased the portion of apoptotic cells (Annexin V-FITC-positive/PI-negative) whereas 250 µM H$_2$O$_2$ relatively increased the portion of late apoptotic cells (Annexin V-FITC-positive/PI-positive) (data not shown). When the effect of H$_2$O$_2$ on MMP ($\Delta$Ψ$_{m}$) in HeLa cells was assessed using Rhodamine 123, H$_2$O$_2$ dose-dependently induced the loss of MMP ($\Delta$Ψ$_{m}$) (Fig. 2D). Examination of apoptosis-related protein changes during H$_2$O$_2$-induced cell death revealed that the level of pro-caspase-3 was decreased by H$_2$O$_2$ (Fig. 2E). The intact 116-kDa form of PARP was decreased by H$_2$O$_2$ whereas the cleaved form was increased (Fig. 2E). Furthermore, autophagy marker light chain 3 (LC3) was converted to LC3-II in the 100 and 250 µM H$_2$O$_2$-treated HeLa cells, indicating that H$_2$O$_2$ induced autophagy in the HeLa cells (Fig. 2E). The activity of caspase-3 was increased in H$_2$O$_2$-treated HeLa cells whereas that of caspase-8 was slightly increased (Fig. 2F).

Effects of caspase inhibitors on apoptosis of H$_2$O$_2$-treated HeLa cells. We investigated whether caspases are required for H$_2$O$_2$-induced apoptosis. Based on a previous study (20), HeLa cells were pretreated with 15 µM of caspase inhibitor for 1 h prior to treatment with H$_2$O$_2$. Treatment with 100 µM H$_2$O$_2$ did not significantly increase the percentages of sub-G1 cells in the HeLa cells at 1, 6 or 12 h, and the pan-caspase inhibitor (Z-VAD) did not affect the percentages at these times (Fig. 3A). H$_2$O$_2$ increased the numbers of Annexin V-FITC-positive cells in the HeLa cells at 6 and 12 h, and Z-VAD markedly reduced the number at 12 h (Fig. 3B). Moreover, treatment with all of the tested caspase inhibitors (Z-VAD, Z-DEVD for caspase-3, Z-IETD for caspase-8 and Z-LEHD for caspase-9) showed the marked rescue of HeLa cells from H$_2$O$_2$-induced cell death at 24 h, as measured by the population of sub-G1 cells (Fig. 3C). In addition, these inhibitors decreased the numbers of Annexin V-FITC-positive cells in the HeLa cells at 6 and 12 h, and Z-VAD markedly increased the number of Annexin V-FITC-positive cells in these cells at 1, 6 and 12 h (data not shown). Z-VAD did not decrease the numbers at these times but instead it increased the number at 12 h (data not shown). In addition, H$_2$O$_2$ increased the numbers of Annexin V-FITC-positive cells in the HeLa cells at 6, 12 and 24 h (data not shown). Z-VAD did not reduce the percentages of Annexin V-FITC-positive cells in the 250 µM H$_2$O$_2$-treated HeLa cells but it increased the number of Annexin V-FITC-positive cells in these cells at
24 h (data not shown). These results indicated that the caspase inhibitors did not protect HeLa cell death induced by 250 μM \( \text{H}_2\text{O}_2 \).

Effects of \( \text{H}_2\text{O}_2 \) on intracellular ROS and GSH levels in HeLa cells. To assess the intracellular ROS levels in the \( \text{H}_2\text{O}_2 \)-treated HeLa cells, \( \text{H}_2\text{DCFDA} \) and DHE dyes were used. All the tested doses of \( \text{H}_2\text{O}_2 \) increased the ROS (DCF) level in the HeLa cells at 24 h (Fig. 4A). The level of DHE fluorescence dye, which specifically reflects \( \text{O}_2^• \) accumulation in cells, was also increased in the \( \text{H}_2\text{O}_2 \)-treated HeLa cells (Fig. 4B). Furthermore, the activities of SOD and catalase in the \( \text{H}_2\text{O}_2 \)-treated HeLa cells were measured. As shown in Fig. 4C, 100 μM \( \text{H}_2\text{O}_2 \) increased the activity of catalase but did not alter the activity of SOD. Following the measurement of intracellular GSH levels in the \( \text{H}_2\text{O}_2 \)-treated HeLa cells using a CMFIDA dye, 100 or 250 μM \( \text{H}_2\text{O}_2 \) increased the GSH-depleted cell number in HeLa cells at 24 h while 50 μM \( \text{H}_2\text{O}_2 \) did not significantly induce GSH depletion (Fig. 4D).

Effects of caspase inhibitors on ROS and GSH levels in the \( \text{H}_2\text{O}_2 \)-treated HeLa cells. To determine whether the levels of intracellular ROS and GSH in the \( \text{H}_2\text{O}_2 \)-treated HeLa cells were altered by treatment with each caspase inhibitor, ROS and GSH levels in the HeLa cells were assessed at the early time point of 1 h and at the extended time point of 24 h (Fig. 5). The intracellular ROS (DCF) level was increased in the \( \text{H}_2\text{O}_2 \)-treated cells at 1 h (Fig. 5A). Z-VAD, caspase-3 and -9 inhibitors seemed to attenuate the increased ROS (DCF) level by \( \text{H}_2\text{O}_2 \), and all the caspase inhibitors decreased the basal level of ROS (DCF) in the HeLa control cells (Fig. 5A). At 24 h, none of the caspase inhibitors significantly affected the ROS (DCF) level in the \( \text{H}_2\text{O}_2 \)-treated HeLa cells (Fig. 5D). Additionally, Z-VAD did not attenuate the increased ROS (DCF) level by \( \text{H}_2\text{O}_2 \) at 6 and 12 h (data not shown). Treatment with 100 μM \( \text{H}_2\text{O}_2 \) did not alter the DHE (\( \text{O}_2^• \)) level in the HeLa cells at 1 h (Fig. 5B). Z-VAD decreased the DHE (\( \text{O}_2^• \)) level in the \( \text{H}_2\text{O}_2 \)-treated and -untreated HeLa cells at 1 h, and other caspase inhibitors reduced the basal level of DHE \( \text{O}_2^• \) in the HeLa control cells (Fig. 5B). In addition, Z-VAD among the caspase inhibitors decreased the DHE (\( \text{O}_2^• \)) level in \( \text{H}_2\text{O}_2 \)-treated HeLa cells at 24 h (Fig. 5E). In regards to the GSH levels, 100 μM \( \text{H}_2\text{O}_2 \) decreased the GSH level in HeLa cells at 1 h (Fig. 5C). Caspase-3 and -9 inhibitors including Z-VAD attenuated the decreased GSH level by \( \text{H}_2\text{O}_2 \), and all inhibitors except the caspase-9 inhibitor reduced the basal level of GSH in the HeLa control cells at 1 h (Fig. 5C). At 24 h, Z-VAD prevented GSH depletion in the \( \text{H}_2\text{O}_2 \)-treated HeLa cells (Fig. 5F).

Discussion

Exogenous \( \text{H}_2\text{O}_2 \) was applied for inducing oxidative stress in HeLa cervical cancer cells. After exposure to \( \text{H}_2\text{O}_2 \) for 24 h, the IC\(_{50}\) value in the HeLa cells was ~75 μM based on MTT assays. \( \text{H}_2\text{O}_2 \) dose-dependently increased the number of dead cells and Annexin V-FITC-positive cells in the HeLa cells, suggesting that \( \text{H}_2\text{O}_2 \)-induced HeLa cell death occurred via apoptosis. Evidently, \( \text{H}_2\text{O}_2 \) decreased the level of procaspase-3 and induced the cleavage of PARP proteins in the HeLa cells. The activity of caspase-3 was also increased in the \( \text{H}_2\text{O}_2 \)-treated HeLa cells. However, 250 μM \( \text{H}_2\text{O}_2 \) did not significantly increase the percentages of sub-G1 cells in the HeLa cells, implying that the relatively higher dose of \( \text{H}_2\text{O}_2 \)
fixed HeLa cells similar to ethanol or methanol. In addition, 100 or 250 µM H$_2$O$_2$ significantly induced LDH release in the HeLa cells at 24 h. Therefore, H$_2$O$_2$ appeared to provoke HeLa cell death via apoptosis as well as necrosis depending on its concentration. Moreover, autophagy appeared to be involved in H$_2$O$_2$-induced HeLa cell death since LC3-I was converted to LC3-II in these cells. Apoptosis is closely related to the collapse of MMP ($\Delta$Ψ$_m$) (24). This result demonstrated that H$_2$O$_2$ triggered the loss of MMP ($\Delta$Ψ$_m$) in HeLa cells in a dose-dependent manner, suggesting that HeLa cell death by H$_2$O$_2$ was tightly correlated with the collapse of MMP ($\Delta$Ψ$_m$). Moreover, it has been reported that ROS may have roles in cell cycle arrest and progression via regulating cell cycle-related proteins (25,26). However, H$_2$O$_2$ did not induce any specific phase arrest of the cell cycle in HeLa cells, suggesting that H$_2$O$_2$-induced oxidative stress did not have an effect on particular proteins related to cell cycle arrest and progression.

Treatment with the caspase inhibitors tested in this experiment significantly prevented HeLa cell death by H$_2$O$_2$, and Z-VAD showed a stronger effect on reducing apoptosis. In particular, although H$_2$O$_2$ slightly increased the activity of caspase-8, its inhibitor significantly prevented HeLa cell death by H$_2$O$_2$. Thus, a subtle change in the activity of caspase-8 seemed to strongly affect the pro-apoptotic pathway in H$_2$O$_2$-treated HeLa cells. These data suggest that the mitochondrial pathway and cell death receptor pathway are together necessary for the complete induction of apoptosis in H$_2$O$_2$-treated HeLa cells. However, Wu et al suggested that H$_2$O$_2$-induced apoptosis in HeLa cells is not through mitochondria-dependent caspase-9 activation (27). The exact apoptotic pathway(s) and the caspase(s) directly involved in the H$_2$O$_2$-induced apoptosis in HeLa cells warrant further studied. With regard to the MMP ($\Delta$Ψ$_m$), caspase inhibitors did not prevent the loss of MMP ($\Delta$Ψ$_m$) induced by H$_2$O$_2$. In addition, caspase inhibitors also did not recover the decreased MMP ($\Delta$Ψ$_m$) level in the H$_2$O$_2$-treated HeLa cells (data not shown). These results imply that the loss of MMP ($\Delta$Ψ$_m$) following treatment with H$_2$O$_2$ activated caspases and consequently induced apoptosis. In addition, the activation of caspase by H$_2$O$_2$ did not positively intensify the MMP ($\Delta$Ψ$_m$) loss. Furthermore, the loss of MMP ($\Delta$Ψ$_m$) by H$_2$O$_2$ may not be enough to fully trigger apoptosis in HeLa cells under the inhibition of caspase activity.

The ROS level was significantly increased in HeLa cells treated with H$_2$O$_2$ at 24 h. Since H$_2$O$_2$ did not decrease the activity of SOD and increased the activity of catalase at 24 h, increases in ROS levels including O$_2^-$ were likely to occur via their strong generation rather than the lack of scavenging them. In addition, it is possible that exogenous H$_2$O$_2$ strongly generates O$_2^-$ via the damage of mitochondria, and both H$_2$O$_2$ and O$_2^-$ can be efficiently converted into the toxic ‘OH via

Figure 5. Effects of caspase inhibitors on ROS and GSH levels in H$_2$O$_2$-treated HeLa cells. Exponentially growing cells were treated with 100 µM H$_2$O$_2$ for 1 or 24 h following 1 h pre-incubation of 15 µM of a caspase inhibitor. ROS and GSH levels in HeLa cells were measured using a FACStar flow cytometer. (A and D) Graphs indicate DCF (ROS) levels (%) at (A) 1 h and (D) 24 h. (B and E) Graphs indicate DHE (O$_2^-$) levels (%) at (B) 1 h and (E) 24 h. (C and F) The graphs indicate mean CMF (GSH) levels (%) at (C) 1 h and (-) CMF (GSH-depleted) cells (%) in HeLa cells compared with control cell group at (F) 24 h. *p<0.05 compared with the control group. #p<0.05 compared with cells treated with H$_2$O$_2$ only. ROS, reactive oxygen species; GSH, glutathione.
the Fenton reaction to kill HeLa cells. However, H$_2$O$_2$ did not increase the O$_2^.$ (DHE) level in HeLa cells at 1 h, suggesting that it did not affect the mitochondrial respiratory transport chain and the activity of various oxidases to generate O$_2^.$ within this early time point. Moreover, caspase inhibitors showing the prevention of H$_2$O$_2$-induced cell death failed to significantly decrease the ROS level including O$_2^.$ at 6, 12 and 24 h. However, Z-VAD, caspase-3 and -8 inhibitors appeared to attenuate the increased ROS (DCF) level by H$_2$O$_2$ at 1 h. In addition, all of the caspase inhibitors decreased the basal level of ROS including O$_2^.$ in the HeLa control cells. It is conceivable that the reduced basal activity of caspase by their inhibitors improves the reliability of antioxidant-related enzymes to strongly scavenge basal intracellular ROS in HeLa cells. Therefore, the early suppression of H$_2$O$_2$-induced oxidative stress by caspase inhibitors seems to be crucial for the protection of HeLa cells against it. The exact role of each caspase inhibitor in preventing H$_2$O$_2$-induced HeLa cell death still needs to be defined further.

GSH is a main non-protein antioxidant in cells. Apoptotic effects are inversely comparable to the GSH content (28-30). Likewise, H$_2$O$_2$ was found to increase the number of GSH-depleted cells in HeLa cells at 24 h. In addition, Z-VAD partially prevented GSH depletion in H$_2$O$_2$-treated HeLa cells. These results support the notion that the intracellular GSH content has a decisive effect on cell death (29,31,32). However, 50 µM H$_2$O$_2$, the dose at which apoptosis is induced in HeLa cells, did not significantly allow GSH depletion in these cells. Moreover, the other caspase inhibitors except Z-VAD failed to prevent GSH depletion in the H$_2$O$_2$-treated HeLa cells. Therefore, the loss of GSH content seemed to be necessary but not sufficient for the induction of apoptosis in the H$_2$O$_2$-treated HeLa cells. Treatment with 100 µM H$_2$O$_2$ decreased the GSH level at 1 h. The decreased GSH level was likely to be due to its use for the decrease in ROS (DCF) level at this time.

In conclusion, H$_2$O$_2$ inhibited the growth of HeLa cells via apoptosis and/or necrosis, which was accompanied by intracellular ROS increase and GSH depletion. The anti-apoptotic effect of caspase inhibitors on H$_2$O$_2$-induced HeLa cell death may result from the early suppression of H$_2$O$_2$-induced oxidative stress. The present data provide useful information for the understanding of the toxicological effect of exogenous H$_2$O$_2$ on HeLa cells.

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