H_2O_2 inhibits the growth of human pulmonary fibroblast cells by inducing cell death, GSH depletion and G1 phase arrest

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Abstract. Cultured normal human cells are invaluable biological models for mechanistic studies of oxidative stress. Exogenous hydrogen peroxide (H_2O_2) is often utilized as a representative model of an oxidative stressor. In the present study, the effect of exogenous H_2O_2 on cell growth and death was evaluated in normal human pulmonary fibroblast (HPF) cells with respect to reactive oxygen species (ROS) and glutathione (GSH) levels. In MTT assays, H_2O_2 inhibited the growth of HPF cells with an IC_{50} of ~50 μM at 24 h. DNA flow cytometric analysis indicated that 50-500 μM H_2O_2 significantly induced G1 phase arrest of the cell cycle. H_2O_2 induced cell death in the HPF cells, which was accompanied by cleavage of caspase-3 and loss of mitochondrial membrane potential (MMP; $\Delta\psi_m$). However, H_2O_2 was not observed to significantly induce sub-G1 cells. H_2O_2 increased superoxide anion ($O_2^{\bullet-}$) levels from 120 min and increases in ROS, including $O_2^{\bullet-}$, were also detected at 24 h. H_2O_2 increased the activity of superoxide dismutase (SOD). H_2O_2 also induced GSH depletion in HPF cells at 24 h and decreased GSH levels after only 25 min. In conclusion, H_2O_2 inhibited the growth of HPF cells via apoptosis and/or necrosis as well as G1 phase arrest, which was accompanied by an intracellular increase in ROS levels and the depletion of GSH. The present study provides an important insight into the toxicological effects of exogenous H_2O_2 on normal HPF cells.

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Abbreviations: HPF, human pulmonary fibroblast; ROS, reactive oxygen species; SOD, superoxide dismutase; 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; GSH, glutathione; CMFDA, 5-chloromethylfluorescein diacetate

Key words: human pulmonary fibroblast, hydrogen peroxide, cell death, reactive oxygen species, glutathione

Introduction

Reactive oxygen species (ROS) are a group of oxygen derivative molecules which include hydrogen peroxide (H_2O_2), the superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$). Compared with other ROS, H_2O_2 freely diffuses through biological membranes to a distance of multiple cell diameters prior to reacting with specific molecular targets. This is due to its solubility in lipid and aqueous environments and its relatively low reactivity. Conventional hypotheses regard ROS to be deleterious or harmful to cells. However, it has become clear that ROS is crucial for the regulation of a number of cellular events, including gene expression, differentiation and cell proliferation (1,2). ROS also function as second messengers, targeting discrete signal transduction pathways in a variety of systems, including the pulmonary system (3,4). ROS are usually generated as by-products of mitochondrial respiration or are specifically produced by oxidases, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase (5). The main metabolic pathways utilize superoxide dismutases (SODs), which metabolize $O_2^{\bullet-}$ to H_2O_2 (6). Further metabolism by catalase or glutathione (GSH) peroxidase, yields O_2 and H_2O (7). Oxidative stress is likely to be the consequence of overproduction of ROS or downregulation of antioxidants, which are associated with cell death (8-10).

The adult human lung is a structurally complex organ system. The epithelial-lined airways and endothelial-lined vasculature are incorporated into an interconnected reticulum of mesenchyme and extracellular matrix (ECM). In particular, fibroblast cells largely derived from primitive mesenchyme, synthesize the ECM and collagen to maintain the structural and functional integrity of connective tissues in the pulmonary system. The lung is vulnerable to various forms of injuries, both airborne and bloodborne, that may result in lung fibrosis and cancer in which fibroblast cells, including myofibroblasts and tumor-associated fibroblasts, are involved (11). For the duration of inflammation, tissue concentrations of H_2O_2 are estimated to reach almost millimolar levels, whereas minute levels of H_2O_2 generated by NADPH oxidase are hypothesized to act only in the microenvironments of the plasma membrane (12,13). However, in both cases H_2O_2 may modulate the essential cellular events of proliferation, differentiation and cell death (apoptosis or necrosis) in fibroblast cells. Exogenous H_2O_2 is commonly considered to be the representative ROS

for modeling oxidative stress. However, the precise molecular mechanisms underlying these important effects in pulmonary fibroblast cells remain obscure. The H_2O_2-induced cytotoxicity in normal fibroblast cells *in vitro* may be of toxicological interest due to the toxic potential of exogenous H_2O_2 in human pulmonary fibroblasts (HPFs).

In the present study, the effects of exogenous H_2O_2 on cell growth and death were evaluated in normal HPF cells with respect to ROS and GSH levels.

Materials and methods

Cell culture. HPF cells from PromoCell GmbH (Heidelberg, Germany) were maintained in a humidified incubator containing 5% CO_2 at 37˚C. The HPF cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin (Gibco-BRL, Grand Island, NY, USA). The cells were grown in 100-mm plastic tissue culture dishes (Nunc A/S, Roskilde, Denmark) containing 10 ml media and harvested with a solution of trypsin-EDTA while in a logarithmic phase of growth. The HPF cells were used between passages 4 and 8.

Cell growth and cell number assays. Cell growth changes in HPF cells treated with H_2O_2 were indirectly determined by measuring the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) dye absorbance. Changes in viable and dead cell counts were determined by trypan blue cell counting. In brief, $5x10^3$ cells/well were seeded in 96-well microtiter plates (Nunc A/S) for MTT assays and $2x10^5$ cells/well were seeded in 24-well plates (Nunc A/S) for cell counting. Following exposure to the indicated amounts of H_2O_2 (Sigma-Aldrich) 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 by trypsin digestion for trypan blue cell counting. 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 cell analysis. Cell cycle and sub-G1 cell analysis were determined by propidium iodide (PI, Ex/Em=488/617 nm; Sigma-Aldrich) staining. In brief, $1x10^6$ cells/60-mm culture dish (Nunc A/S) were incubated with the indicated concentrations of H_2O_2 for 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, 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 by Lysis II and Cellfit software (Becton-Dickinson).

Annexin V-fluorescein isothiocyanate (FITC) staining for cell death detection. Apoptosis was determined by staining the HPF cells with annexin V-FITC (Invitrogen Life Technologies, Camarillo, CA, USA; Ex/Em=488/519 nm). In brief, $1x10^6$ cells/60-mm culture dish were incubated with the indicated concentrations of H_2O_2 for 24 h. 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 and 2.5 mM $CaCl_2$] at a concentration of $1x10^6$ cells/ml. Annexin V-FITC (5 μl) was then added and the cells analyzed with a FACStar flow cytometer.

Western blot analysis. Caspase-3 protein expression in H_2O_2-treated cells was determined by western blot analysis. In brief, $1x10^6$ cells/60-mm culture dish were incubated with 50 μM H_2O_2 for 24 h. Cells were then washed with 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) NP40, 0.5 mM DTT and 1% (v/v) protease inhibitor cocktail]. Supernatant protein concentrations were determined using the Bradford method. Samples containing 10 μg total protein were resolved by 12.5% SDS-PAGE gels, transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA, USA) by electroblotting and then probed with anti-caspase-3 and anti-β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and blots were developed using an ECL kit (Amersham, Arlington Heights, IL, USA).

Measurement of mitochondrial membrane potential [MMP ($\Delta\psi_m$)]. The MMP ($\Delta\psi_m$) levels were measured using a rhodamine 123 fluorescent dye (Sigma-Aldrich; Ex/Em=485/535 nm). In brief, $1x10^6$ cells/60-mm culture dish were incubated with the indicated concentrations of H_2O_2 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 using a FACStar flow cytometer. The cells that were rhodamine 123-negative were indicated to have lost MMP ($\Delta\psi_m$).

Detection of intracellular ROS levels. Intracellular ROS were detected using a fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Ex/Em=495/529 nm; Invitrogen Life Technologies) at the indicated times. H_2DCFDA is poorly selective for $O_2^{\bullet-}$. By contrast, dihydroethidium (DHE, Ex/Em=518/605 nm; Invitrogen Life Technologies) is a fluorogenic probe that is highly selective for $O_2^{\bullet-}$ among ROS. In brief, $1x10^6$ cells/ml in a FACS tube (Becton-Dickinson) were treated with the indicated concentrations of H_2O_2 in the presence of 20 μM H_2DCFDA or DHE. Levels of DCF and DHE fluorescence were evaluated using a FACStar flow cytometer at the indicated times. DCF (ROS) and DHE ($O_2^{\bullet-}$) levels were expressed as mean fluorescence intensity (MFI), which was calculated using CellQuest software (Becton-Dickinson). In addition, $1x10^6$ cells/60-mm culture dish were incubated with the indicated concentrations of H_2O_2 for 24 h. 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.

Measurement of cellular SOD and catalase activities. SOD enzyme activity was measured using the SOD Assay kit-WST (Fluka Chemical Corp., Milwaukee, WI, USA) and catalase

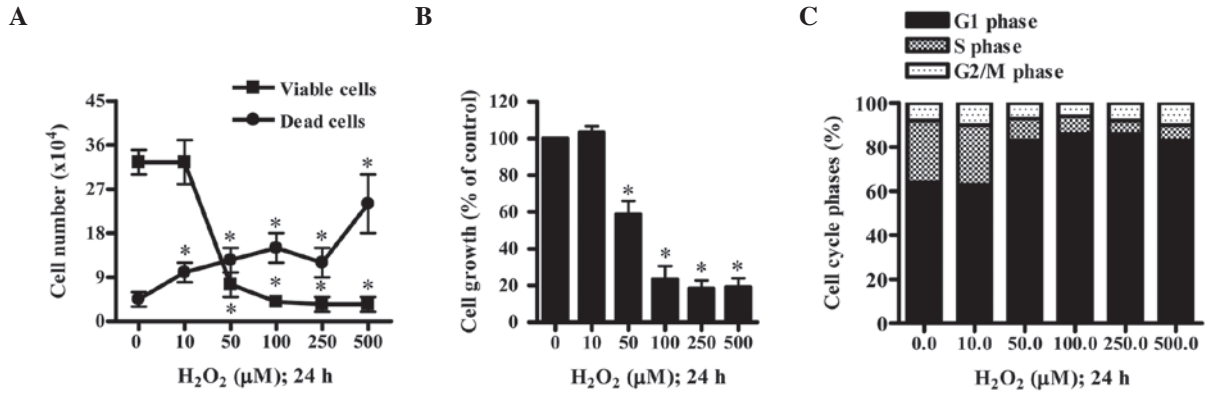

Figure 1. Effect of H_2O_2 on HPF cell growth. Exponentially growing cells were treated with the indicated concentrations of H_2O_2 for 24 h. Graphs present (A) viable (trypan blue-negative) and dead (trypan blue-positive) cell numbers of HPF cells, (B) precellular growth changes in HPF cells, as assessed by MTT assays and (C) cell cycle distributions of the HPF cells, as measured by FACStar flow cytometer. *P<0.05, vs. control group. H_2O_2, hydrogen peroxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HPF, human pulmonary fibroblast.

enzyme activity was measured using the Catalase assay kit from Sigma-Aldrich. In brief, $1x10^6$ cells were incubated with 50 μM H_2O_2 for 24 h. Cells were then washed with 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 and 1% protease inhibitor cocktail (Sigma-Aldrich)]. The supernatant protein concentration was determined by the Bradford method. Supernatant samples containing 100 μg total protein were used for determination of SOD and catalase enzyme activities. The samples were added to each well in 96-well microtiter plates with the appropriate working solutions (according to the manufacturer's instructions) at 25˚C for 30 min. Color changes were measured at 450 or 520 nm using a microplate reader (Spectra MAX 340, Molecular Devices, LLC, Sunnyvale, CA, USA). The value for the experimental group was converted to a percentage of that of the control group.

Detection of intracellular GSH. Cellular GSH levels were analyzed using a 5-chloromethylfluorescein diacetate dye (CMFDA, Ex/Em=522/595 nm; Invitrogen Life Technologies) at the indicated times or 24 h. In brief, $1x10^6$ cells/ml in a FACS tube (Becton-Dickinson) were treated with the indicated concentrations of H_2O_2 in the presence of 5 μM CMFDA. The level of CMF fluorescence was evaluated using a FACStar flow cytometer at the indicated early times. CMF (GSH) levels were expressed as the MFI, which was calculated using CellQuest software (Becton-Dickinson). In addition, $1x10^6$ cells in a 60-mm culture dish were incubated with the indicated amounts of H_2O_2 for 24 h. Cells were 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 represent the mean of at least two independent experiments (mean ± SD). Data were analyzed using Instat software (GraphPad Prism4, San Diego, CA, USA). The 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

Effect of H_2O_2 on HPF cell growth. The effect of H_2O_2 on the growth of HPF cells was examined at 24 h. While 10 μM H_2O_2 did not alter the viable (trypan blue-negative) cell number of the HPF cells, 50-500 μM H_2O_2 significantly decreased the population of viable HPF cells in a concentration-dependent manner (Fig. 1A). In addition, H_2O_2 increased the number of dead (trypan blue-positive) cells in a concentration-dependent manner (Fig. 1A). The ratio of dead cells to viable cells was increased by H_2O_2 treatment. Based on MTT assays, 10 μM H_2O_2 did not inhibit HPF cell growth whereas 50-500 μM H_2O_2 was observed to significantly inhibit the growth of HPF cells with an IC_{50} (the half maximal inhibitory concentration) of ~50 μM (Fig. 1B). When the cell cycle distributions of the H_2O_2-treated HPF cells were examined, 50-500 μM H_2O_2 was identified to significantly induce G1 phase arrest compared with that of control cells (Fig. 1C).

Effect of H_2O_2 on cell death and MMP ($\Delta\psi_m$) in HPF cells. Next, we determined whether H_2O_2 induced cell death via apoptosis in HPF cells. The tested concentrations of H_2O_2 did not increase the percentage of sub-G1 HPF cells, which implied that dead cells in H_2O_2-treated HPF cells were not converted into sub-G1 cells (Fig. 2A). Treatment with 50-500 μM H_2O_2 increased the percentage of annexin V-FITC-positive HPF cells in a concentration-dependent manner (Fig. 2B). H_2O_2 decreased procaspase-3 in HPF cells, which indirectly demonstrated that H_2O_2 activated caspase-3 in these cells (Fig. 2B). Since apoptosis is closely associated with the collapse of MMP ($\Delta\psi_m$) (14), the effect of H_2O_2 on MMP ($\Delta\psi_m$) was assessed in HPF cells using rhodamine 123. Treatment with 50-500 μM H_2O_2 was identified to significantly induce the loss of MMP ($\Delta\psi_m$) in HPF cells (Fig 2C).

Effect of H_2O_2 on intracellular ROS levels in HPF cells. To assess the intracellular ROS levels in H_2O_2-treated HPF cells, H_2DCFDA and DHE dyes were utilized. Treatment with 50 or 100 μM H_2O_2 increased ROS (DCF) levels from 25 min and 10 μM H_2O_2 also increased levels from 90 min (Fig. 3A). A gradual increase in ROS (DCF) levels was observed up

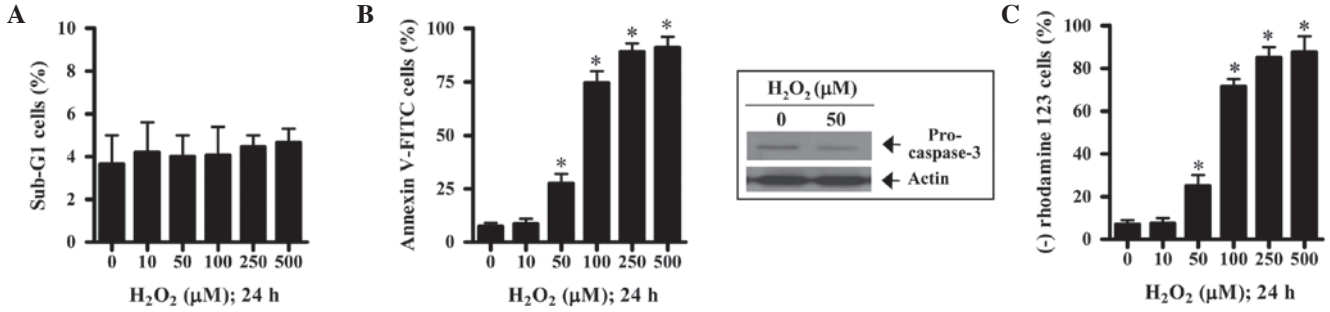

Figure 2. Effect of H_2O_2 on cell death and MMP ($\Delta\psi_m$) in HPF cells. Exponentially growing cells were treated with the indicated concentrations of H_2O_2 for 24 h. Graphs present (A) percentages of sub-G1 HPF cells and (B) percentages of annexin V-FITC-positive cells, as measured by FACStar flow cytometer. Western blot analysis indicates the levels of procaspase-3 in 50 μM H_2O_2-treated HPF cells. (C) Graph reveals the percentages of rhodamine 123-negative [MMP ($\Delta\psi_m$) loss] cells. *P<0.05, vs. control group. H_2O_2, hydrogen peroxide; MMP, mitochondrial membrane potential; FITC, fluorescein isothiocyanate; HPF, human pulmonary fibroblast.

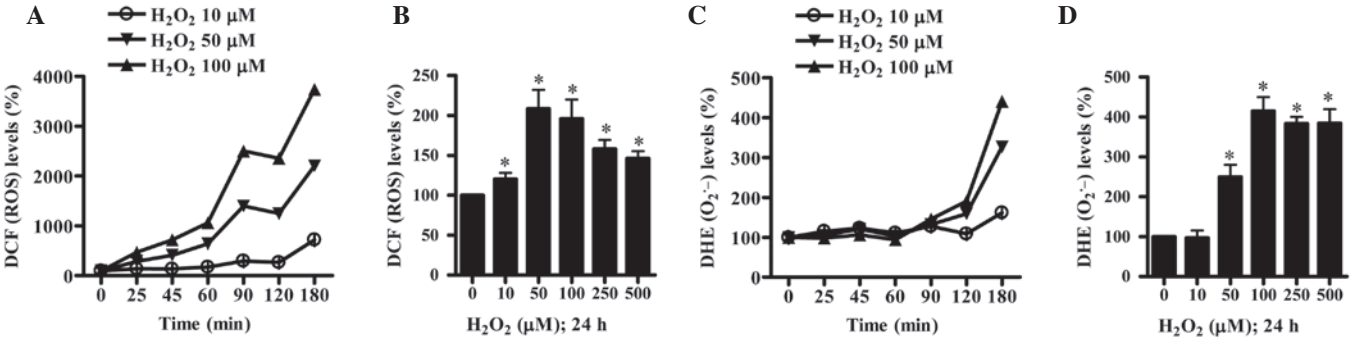

Figure 3. Effect of H_2O_2 on ROS levels in HPF cells. Exponentially growing cells were treated with the indicated concentrations of H_2O_2 for specific durations. ROS levels in HPF cells were measured using a FACStar flow cytometer. Graphs present (A and B) DCF (ROS) and (C and D) DHE ($O_2^{\bullet-}$) levels (%) in HPF cells compared with control. *P<0.05, vs. control group. H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; HPF, human pulmonary fibroblast; DCF, 2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; $O_2^{\bullet-}$, superoxide anion radical.

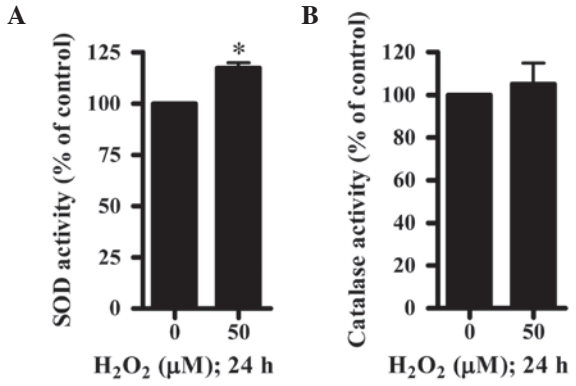

Figure 4. Effect of H_2O_2 on the activities of SOD and catalase in HPF cells. Exponentially growing cells were treated with 50 μM H_2O_2 for 24 h. Activities of SOD and catalase were measured. Graphs demonstrate changes in (A) SOD and (B) catalase activities. *P<0.05, vs. control group. H_2O_2, hydrogen peroxide; HPF, human pulmonary fibroblast; SOD, superoxide dismutase.

to 180 min, however, there was a transient decrease in ROS levels at 120 min (Fig. 3A). In addition, all tested concentrations of H_2O_2 increased ROS (DCF) levels in HPF cells at 24 h and the level in the 50 μM H_2O_2-treated HPF cells was the highest (Fig. 3B). The level of DHE fluorescent dye, which specifically reflects $O_2^{\bullet-}$ accumulation in cells, was also increased in 50 or 100 μM H_2O_2-treated HPF cells from 120 min (Fig. 3C). Treatment with 10 μM H_2O_2 increased $O_2^{\bullet-}$ levels at 180 min (Fig. 3C). Treatment with 50-500 μM H_2O_2 significantly increased $O_2^{\bullet-}$ levels at 24 h whereas 10 μM H_2O_2 did not alter these levels (Fig. 3D). Furthermore, the activities of SOD and catalase were measured in H_2O_2-treated HPF cells. As demonstrated in Fig. 4, 50 μM H_2O_2 increased the activity of SOD but did not alter the activity of catalase.

Effect of H_2O_2 on GSH levels in HPF cells. The measurement of intracellular GSH levels in H_2O_2-treated HPF cells using a CMFDA dye revealed that 50 or 100 μM H_2O_2 decreased GSH (CMF) levels by ~30% compared with the control at 25 min (Fig. 5A). The levels gradually recovered up to 120 min and then markedly decreased again at 180 min (Fig. 5A). Treatment with 10 μM H_2O_2 also decreased GSH (CMF) levels at 60 or 180 min but transiently increased levels at 120 min (Fig. 5A). Furthermore, 50-500 μM H_2O_2 significantly increased GSH depleted cell numbers in HPF cells at 24 h (Fig. 5B).

Discussion

Cultured normal human cells are invaluable biological models for mechanistic studies of genotoxic or cytotoxic chemicals and drugs. The present study focused on elucidating the cytotoxic effect of exogenous H_2O_2 on cell growth and death in normal HPF cells with respect to changes in ROS and GSH

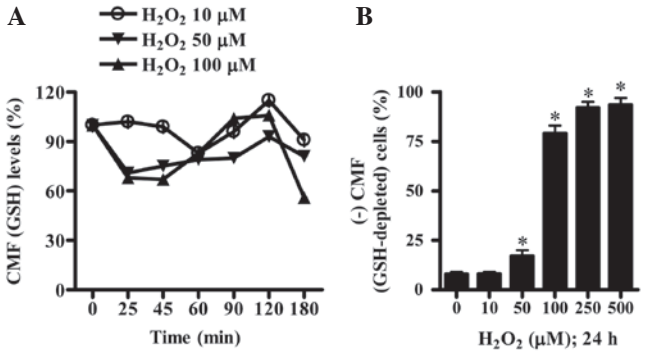

Figure 5. Effect of H_2O_2 on GSH levels in HPF cells. Exponentially growing cells were treated with the indicated concentrations of H_2O_2 for specific durations. GSH levels in HPF cells were measured using a FACStar flow cytometer. Graphs indicate (A) CMF (GSH) and (B) (-) CMF (GSH-depleted) cells (%) at 24 h in HPF cells compared with control. *P<0.05, vs. control group. H_2O_2, hydrogen peroxide; GSH, glutathione; HPF, human pulmonary fibroblast; CMF, 5-chloromethylfluorescein.

levels. Exposure to H_2O_2 for 24 h inhibited the growth of HPF cells with an IC_{50} of ~50 μM. According to our unpublished data, H_2O_2 reduced the growth of human pulmonary artery smooth muscle (HPASM) cells with an IC_{50} of ~250-500 μM at 24 h. These results indicate that the susceptibility of HPF cells to H_2O_2 is high compared with that of HPASM cells. Thus, the cytotoxic effects of H_2O_2 differ among various normal lung cells depending on their origins. In addition, H_2O_2 inhibited the growth of Calu-6 and A549 lung cancer cells with IC_{50} values of ~75 and 10 μM at 24 h, respectively (unpublished data). HPF cells appear to be extremely vulnerable to oxidative stress compared with other lung cells, including cancer cells. It is hypothesized that HPF cells, among other lung-related cells, may be primarily and frequently involved in lung inflammation and fibrosis.

H_2O_2 increased the percentage of annexin V-FITC-positive cells in the HPF cell population, indicating that H_2O_2-induced HPF cell death occurred via apoptosis. H_2O_2 also activated caspase-3 in the cells. Notably, none of the tested concentrations of H_2O_2 significantly increased the number of sub-G1 cells, implying that H_2O_2 fixed HPF cells in a similar manner to ethanol or methanol. Therefore, H_2O_2 appeared to provoke HPF cell death via apoptosis as well as necrosis. In addition, H_2O_2 induced MMP ($\Delta\psi_m$) loss in a concentration-dependent manner in HPF cells. The levels of MMP ($\Delta\psi_m$) loss were comparable with the levels of annexin V-stained cells, indicating that H_2O_2-induced cell death is associated with the collapse of MMP ($\Delta\psi_m$). Furthermore, DNA flow cytometric analysis indicated that 50-500 μM H_2O_2 significantly induced a G1 phase arrest of the cell cycle in HPF cells. Thus, G1 phase arrest in H_2O_2-treated HPF cells was an underlying mechanism suppressing the growth of HPF cells along with cell death.

The main ROS involved in cell signaling pathways are H_2O_2 and $O_2^{\bullet-}$. ROS toxicity is usually mediated by $^{\bullet}OH$ (4). The current results indicate that ROS levels, including those of $O_2^{\bullet-}$, were significantly increased in the HPFs treated with H_2O_2. However, the ROS types and concentrations varied depending on the incubation times and concentrations of H_2O_2. Treatment with 50 or 100 μM H_2O_2 increased ROS (DCF) levels from 25 min whereas 10 μM H_2O_2 increased levels from 90 min. Although there was a transient decrease in ROS levels at 120 min during this gradual increase, the increased levels exceeded baseline. DHE ($O_2^{\bullet-}$) levels in 50 and 100 μM H_2O_2-treated HPF cells were increased from 120 min and from 180 min in 10 μM H_2O_2-treated HPF cells. H_2O_2 was observed to damage the mitochondria, particularly the mitochondrial respiratory transport chain, from 25 min. This damage led to induction of electron leakage from the chain and increased DHE ($O_2^{\bullet-}$) levels at 120 min. It is possible that H_2O_2 also activated oxidases, including NADPH oxidase and xanthine oxidase, in HPF cells to generate $O_2^{\bullet-}$ and increased the activity of SOD. Thus, H_2O_2 appeared to increase the DHE ($O_2^{\bullet-}$) level via ROS generation rather than scavenging. All concentrations of H_2O_2 were identified to increase ROS (DCF) levels in HPF cells at 24 h and 50 μM H_2O_2 was observed to be the most potent. However, 10 μM H_2O_2 did not increase DHE ($O_2^{\bullet-}$) levels at 24 h and 100-500 μM H_2O_2 markedly increased levels of DHE ($O_2^{\bullet-}$) compared with ROS (DCF). Since H_2O_2 concentrations >50 μM induced cell death and MMP ($\Delta\psi_m$) loss in HPF cells, it is possible that exogenous H_2O_2 markedly generates $O_2^{\bullet-}$ by mitochondrial damage and H_2O_2 and $O_2^{\bullet-}$ are efficiently converted into toxic $^{\bullet}OH$ via the Fenton reaction to kill HPF cells.

Apoptotic effects are inversely correlated with GSH content (15). Similarly, H_2O_2 increased the number of GSH-depleted cells in HPF at 24 h, which correlated with annexin V-FITC results from the HPF cells treated with H_2O_2. These results support the hypothesis that intracellular GSH content has a decisive effect on cell death (15). Treatment with 50 and 100 μM H_2O_2 decreased GSH levels at 25 min and the levels were partially recovered until 120 min. Following this, GSH levels were markedly reduced at 180 min. Since H_2O_2 markedly increased ROS (DCF) levels at 25 min, GSH levels were expected to decrease in order to reduce ROS (DCF) levels. In addition, as DHE ($O_2^{\bullet-}$) levels in the H_2O_2-treated HPF cells increased from 120 min, GSH levels were observed to markedly decrease at 180 min due to increased ROS levels, including $O_2^{\bullet-}$. Thus, GSH levels correlate with ROS levels in H_2O_2-treated HPF cells and are also differently affected by various species of ROS.

In conclusion, H_2O_2 inhibited HPF cells growth via apoptosis and/or necrosis as well as G1 phase arrest, which was accompanied by increased intracellular ROS levels and GSH depletion. HPF cells appear to be extremely vulnerable to exogenous H_2O_2 compared with other lung cells, including cancer cells. The present studies provides an important insight into the toxicological effect of exogenous H_2O_2 on normal HPF cells.

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