Abstract. Background: Oxidative stress has been shown to play an important role in cancer progression. In lung cancer, increasing expression of caveolin-1 (Cav-1) has been found in both primary and metastatic carcinomas and may be critical in the regulation of the oxidative status of cancer cells. Materials and Methods: Using molecular and pharmacological manipulations, the role of Cav-1 in regulating cellular oxidative status in lung cancer cells was investigated. The level of Cav-1 was determined by western blot analysis and reactive oxygen species (ROS) were detected by specific fluorescence probes. Results: The treatment of lung cancer H460 cells with hydrogen peroxide (H2O2) significantly up-regulated ROS inside the cells and contributed to cell apoptosis. While cells stably transfected with Cav-1 overexpressing plasmids (H460/Cav-1) exhibited decreased ROS signal and attenuated cell death rate, shRNACav-1 transfected (H460/shCav-1) cells showed enhanced ROS signal and increased cell damage. The use of specific superoxide anion and the hydrogen peroxide detecting assays and hydroxyl radical inhibition assay indicated that the variable oxidative stress found in these cells was mainly due to the alteration of the cellular hydroxyl radical level. Conclusion: A novel role of Cav-1 protein is the suppression of cellular oxidative stress induced by H2O2.

Abbreviations: CAT, catalase; Cav-1, caveolin-1; DCFH2-DA, dichlorofluorescein diacetate; DFO, deferoxamine; DHE dihydroethidium; GSH, glutathione; H2O2, hydrogen peroxide; HPF, 3’-(p-hydroxyphenyl) fluorescein; MnTBAP, Mn(III)tetrakis (4-benzoic acid) porphyrin chloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetylcysteine; PI, propidium iodide.

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Although reactive oxygen species (ROS) are widely accepted to be important mediators of normal cell processes, when in excess, they cause oxidative stress implicated in the damage of cellular components and subsequently cell death (1-3). Such cell and tissue damage has been shown to be associated with several pathological conditions (4-6). In cancer, ROS are considered as carcinogens since evidence has indicated their roles in facilitating carcinogenesis and tumor progression (5, 7-10), and they also affect cancer cell survival and behavior (11, 12). Chronic inflammation as found in the environments of several human carcinomas including lung cancer, is likely to be a major cause of increased ROS in such microenvironments (5, 13). Indeed, ROS, including superoxide anions, hydrogen peroxide (H2O2) and singlet oxygen, are released from inflammatory cells of the innate immune system, and cause oxidative damage to surrounding cancer cells (13, 14). Several studies have documented high ROS levels in the lung of lung cancer patients (15, 16).

In certain types of tumor including lung cancer, an up-regulation of caveolin-1 (Cav-1), a major protein component of caveolae, has been observed (17, 18) and the Cav-1 protein was shown to mediate anoikis resistance and other aggressiveness behaviors such as migration and invasion of cancer cells (19-21). Moreover, the expression of Cav-1 has been related to poor prognosis in lung (22) and prostate carcinomas (23, 24). Cav-1 has been shown to function as a scaffolding protein (25) and to regulate certain proteins such as Src-like kinases, endothelial nitric oxide, and H-Ras (26-30). Although the contributions of Cav-1 protein on cancer progression and metastasis have been intensively investigated and reported, information regarding the role of Cav-1 in controlling redox status and in oxidative stress-induced damage is still limited.

Among key ROS presenting in physiological and pathological conditions, H2O2 has gained the most attention since it is relatively stable in comparison to other principle ROS and can pass through biological membranes and spread in tissues (31, 32). In cancer microenvironments, an increase of ROS, especially H2O2, has been well recognized (16, 33).
Although the role of H₂O₂ in cancer cell viability either in promoting cell survival or inducing cell death is controversial, many researchers believe that H₂O₂ at basal level and low concentrations could favor cell proliferation and viability (2, 8), whereas at high doses, H₂O₂ could induce cell damage through the induction of intracellular oxidative stress (33, 34). According to this concept, cancer cells immersing in H₂O₂-rich microenvironments may possess mechanisms which allow them to survive.

Cav-1 protein could play a role in modulating the cellular oxidative condition. The present study investigated the role of Cav-1 protein in H₂O₂-induced cell death in human lung carcinoma H460 cells, using molecular and pharmacological manipulations.

Materials and Methods

Cells and reagents. Human non-small cell lung cancer H460 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultivated in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 units/ml penicillin/streptomycin in a 5% CO₂ environment at 37°C. N-acetylcysteine (NAC), reduced glutathione (GSH), sodium pyruvate, H₂O₂, catalase (CAT), Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), propidium iodide (PI), Hoechst 33342, 2',7'-dichlorofluorescin diacetate (DCFH₂-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and deoxyrribonucleic acid (DNA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dihydroethidium (DHE) and 3'-(p-hydroxyphenyl) fluorescein (HPF) from Molecular Probes, Inc. (Eugene, OR, USA); antibody for Cav-1 and peroxidase-conjugated secondary antibody from Abcam (Cambridge, MA, USA) and the transfecting agent Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA).

Plasmid and transfection. The Cav-1 overexpressed (H460/Cav-1) and Cav-1 knockdown (H460/shCav-1) cells were established by transfection of the H460 cells with Cav-1 plasmids obtained from the American Type Culture Collection and Cav-1 knockdown plasmid shRNA-Cav-1 obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Briefly, 60% confluent cells were transfected with 15 μl of Lipofectamine 2000 reagent and 2 μg of Cav-1, shRNA-Cav-1 or mock control plasmids. After 16 h, the medium was replaced with culture medium containing 10% fetal bovine serum. Approximately 3 days after the beginning of transfection, the single cell suspensions were plated onto 75 ml culture flasks and cultured for 60 days with an antibiotic selection. The expression of Cav-1 in the transfectants was quantified by western blot analysis.

ROS detection. Intracellular ROS were determined using the ROS-specific probe, DCFH₂-DA, superoxide anions were determined by DHE, and hydroxyl radicals were determined by HPF. The cells were incubated with 10 μM of DCFH₂-DA, H₂O₂ or DHE for 30 min at 4°C, after which they were washed and immediately analyzed for fluorescence intensity by fluorescence microplate reader (SpectraMax M5, Molecular Devices Corp., Sunnyvale, CA, USA) using a 480-nm excitation beam and a 530-nm band-pass filter for detecting DCF fluorescence, using a 490-nm excitation beam and a 515-nm band-pass filter for HPF or using a 488-nm excitation beam and a 610-nm band-pass filter for DHE, and visualized under a fluorescence microscope (Eclipse Ti-U, Nikon, Tokyo, Japan).

Cytotoxicity assay. To determine H₂O₂-mediated cytotoxicity, cell viability was determined by MTT assay. After the specified treatment, the cells were incubated with 10 μM Hoechst and 5 μg/ml PI dye for 30 min at 37°C. All the analyses were performed in at least three independent replicate cultures. The absorbance ratio of treated to non-treated cells was calculated and presented as relative cell viability.

Apoptosis and necrosis assay. Apoptotic and necrotic cell death was determined by Hoechst 33342 and PI co-staining. After the specified treatments, the cells were incubated with 10 μM Hoechst and 5 μg/ml PI dye for 30 min at 37°C. The apoptotic cells having condensed chromatin and/or fragmented nuclei and PI-positive necrotic cells were visualized and scored under a fluorescence microscope.

Western blotting. Cell lysates were obtained by incubating the cells in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 150 mM sodium chloride, 1% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 60 min on ice. The protein content was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) and equal protein samples (30 μg) were heated at 95°C for 5 min with Laemmli loading buffer. Then, the lysates were loaded on 10% SDS-polyacrylamide gel for electrophoresis. After separation, the proteins were transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 h in 5% non-fat dry milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween 20) and incubated with the anti-Cav-1 antibodies at 4°C for 10 h. The membranes were washed twice with TBST for 15 min and incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies for 2 h at room temperature. The immune complexes were detected by enhanced chemiluminescence substrate (Supersignal West Pico; Pierce, Rockford, IL, USA) and quantified using analyst/PC densitometry software (Bio-Rad).

Statistical analysis. The mean data from at least three independent experiments were normalized to the results of the non-treated control. Statistical differences between the means were determined using an analysis of variance (ANOVA) and post hoc test at a significance level of p<0.05, and presented as the mean±S.D.

Results

Effect of hydrogen peroxide on oxidative stress and cell death. Subconfluent (90%) monolayers of H460 cells were treated with H₂O₂ (0–200 μM) for various times and ROS levels were analyzed using DCFH₂-DA as the probe. Figure 1a and b show that H₂O₂ exposure caused a dose-dependent increase in cellular DCF fluorescence intensity as early as 10 min after the start of treatment and a steady cellular ROS signal was observed after 70 min. While the control non-treated H460
cells exhibited no significant change in cellular ROS content throughout the time of detection, treatment with 100-200 μM H₂O₂ caused significantly up-regulated intracellular ROS signals. Notably, the treatment with 200 μM H₂O₂ exhibited a dramatic increase in the ROS signal with approximately 2.75-fold induction in cellular ROS compared to a non-treated control. The MTT assay showed that this H₂O₂ dose caused a significant reduction of cell survival with decreasing cell viability as early as 3 h after treatment and the reduction further continued until 24 h with approximately 50% of the cells remaining viable (Figure 1c and e). An analysis of cell apoptosis by Hoechst 33342 nuclear staining assay further revealed that the decrease of cell viability was due to apoptosis, as indicated by the increasing number of cells with condensed nuclear fluorescence and nuclear fragmentation (Figure 1d and f), whereas PI-positive cells, indicating necrotic cells, were not detected under these conditions.

Specific reactive oxygen species up-regulation in response to hydrogen peroxide treatment. The H460 cells were pretreated with pan- or specific-antioxidants, namely NAC, GSH, catalase, sodium pyruvate, DFO or MnTBAP for 1 h followed by H₂O₂ treatment (200 μM) for 24 h. The treatment with NAC and GSH, pan-antioxidants, significantly preserved viability of the H₂O₂-treated H460 cells (Figure 2a). The H₂O₂-scavengers catalase and sodium pyruvate and the hydroxyl radical inhibitor DFO also significantly prevented the cytotoxic effect of H₂O₂, whereas MnTBAP, a superoxide dismutase mimetic, had no protective effect. Confirmation studies using the DCFH-2-DA probe for ROS evaluation further showed a decrease of oxidative signal in all the antioxidant pre-treated cells excepted for the MnTBAP treated cells (Figure 2b).
Importantly, administration of DFO prior to H₂O₂ could solely protect cell damage and completely prevented the induction of a DCF signal in response to H₂O₂. When the cells were treated with H₂O₂ in the presence of DCFH₂-DA for ROS detection, DHE for superoxide anion detection or HPF for hydroxyl radical detection, significantly increased in DCF and HPF signals occurred, but no effect on DHE signal was found (Figure 2a).

Effect of caveolin-1 on hydrogen peroxide-induced oxidative stress and cell death. Western blot analysis of Cav-1 expression in the stably transfected H460 cells showed a substantial increase in Cav-1 level in the H460/Cav-1 cells and significantly reduced Cav-1 level in the H460/shCav-1 cells (Figure 3a). When the cells were treated with 200 μM H₂O₂, a significantly higher ROS signal was found in the H460/shCav-1 cells in comparison to the parental H460 cells, while the H460/Cav-1 cells had a lower ROS signal compared to the H460 and H460/shCav-1 cells (Figure 3b and c). H460, H460/Cav-1 and H460/shCav-1 cells were also treated with H₂O₂ in the presence of the hydroxyl radical probe and as expected, the H460/shCav-1 cells exhibited the highest level of hydroxyl radical signal, whereas the H460/Cav-1 cells expressed the lowest level (Figure 3d).

To substantiate the role of Cav-1 in the regulation of oxidative stress-induced damage to these cells, H460, H460/Cav-1 and H460/shCav-1 cells were treated with 200 μM H₂O₂, and cell viability was verified by MTT assay after 24 h. The Cav-1 transfected cells exhibited a higher survival rate, while the shRNA-Cav-1 transfected cells showed a lower percentage of cell survival over time as compared with the parental H460 cells (Figure 3e). At 24 h post-treatment, the H460/Cav-1 cells exhibited ~85% viability, whereas both the H460 and H460/shCav-1 cells showed a survival rate of <50%.
Effect of deferoxamine and iron on H\textsubscript{2}O\textsubscript{2}-mediated oxidative stress and cell death. Figure 4 shows that treatment of the H460, H460/Cav-1 and H460/shCav-1 cells with 200 μM H\textsubscript{2}O\textsubscript{2} for 24 h caused a significant decrease in cell survival in all the cells. The H460/shCav-1 cells exhibited the highest reduction in viability rate, whereas the H460/Cav-1 cells expressed the lowest rate of cell death. The addition of the hydroxyl radical inhibitor DFO significantly protected against cell death induced by H\textsubscript{2}O\textsubscript{2} in all the cells. The H460/shCav-1 cells exhibited the highest reduction in viability rate, whereas the H460/Cav-1 cells expressed the lowest rate of cell death. The addition of the hydroxyl radical inhibitor DFO significantly protected against cell death induced by H\textsubscript{2}O\textsubscript{2} in all the cells. These observations were confirmed by the treatment of the cells with iron, which dramatically enhanced the cell death response to H\textsubscript{2}O\textsubscript{2} in all the cells. Also, DCFH\textsubscript{2}-DA and HPF probes were used for the detection of cellular ROS and hydroxyl radicals, respectively. Treatment of the H460, H460/Cav-1 and H460/shCav-1 cells with DFO successfully inhibited both ROS and hydroxyl radical inductions caused by H\textsubscript{2}O\textsubscript{2} in all the cells (Figure 5a and c), whereas the treatment of these cells with iron caused a dramatic increase in both ROS (Figure 5b) and hydroxyl radical levels (Figure 5d).

**Discussion**

Cellular oxidative stress as well as cytotoxicity caused by H\textsubscript{2}O\textsubscript{2} exposure were attenuated in Cav-1 overexpressing lung cancer cells while shRNA-mediated Cav-1 down-regulated cells were highly susceptible to H\textsubscript{2}O\textsubscript{2}-induced cell damage. It is well documented that H\textsubscript{2}O\textsubscript{2} can be detoxified to water by cellular antioxidant enzymes including glutathione peroxidase and catalase (1, 35), however, in the presence of reduced transition metal ions such as iron or copper, H\textsubscript{2}O\textsubscript{2} can be rapidly converted to highly reactive hydroxyl radicals.
through Fenton-like reactions (1, 36).

The H$_2$O$_2$ treatment of the cells in the present study resulted in an induction of cellular ROS, namely H$_2$O$_2$ and hydroxyl radicals. Increasing evidence has indicated different roles of specific ROS in regulation of cell behaviors (2, 8, 33, 37). Our previous experiments indicated that superoxide anions and H$_2$O$_2$ inhibited lung cancer cell migration and invasion whereas hydroxyl radicals had opposite effects (20). Also, endogenous H$_2$O$_2$, but not hydroxyl radicals could render cancer cells resistant to detachment-induced apoptosis (21). Previous experiments indicated that H$_2$O$_2$ generated in response to cisplatin treatment mediated renal cell necrosis, whereas hydroxyl radicals played a principle role in apoptosis induction (37). This finding was consistent with the present observation that blocking of hydroxyl radical formation by DFO pre-treatment dramatically inhibited H$_2$O$_2$-induced cell death in the H460 cells (Figure 4).

The cells stably transfected with Cav-1 overexpressing plasmids, showed a marked increase of Cav-1 level over the one of the parental H460 cells and the shRNA-transfected cells showed a dramatically reduced Cav-1 level (Figure 3a). Furthermore, Cav-1 functioned in attenuating the H$_2$O$_2$-derived hydroxyl radicals in the H460 cells and reduced H$_2$O$_2$-mediated death. In some studies, Cav-1 has been shown to sensitize cancer cells to apoptosis in response to death stimuli and a reduction of Cav-1 level has been shown to contribute to chemotherapeutic cisplatin and carboplatin resistance (38). Furthermore, we have recently reported that Cav-1 sensitizes cisplatin-induced cell death through superoxide anion induction (39). In contrast, the present study revealed that only minimal change in the cellular superoxide anion level occurred in response to H$_2$O$_2$ treatment (Figure 2c) and Cav-1 overexpression was able to protect against cell death. Although further investigations are necessary to clarify the mechanism(s) by which Cav-1 possess such opposite effects on the specific ROS mechanisms of cells, the present study has revealed for the first time that Cav-1 could attenuate the oxidative stress induced by H$_2$O$_2$.

In conclusion, Cav-1 modifies cellular oxidative stress induced by H$_2$O$_2$ treatment and renders non-small cell lung cancer cell resistant to apoptosis. Since sustained or elevated Cav-1 expression and H$_2$O$_2$ level could be concomitantly found in certain carcinomas, especially lung cancer, these findings may help providing better understanding of cancer biology.

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References

Figure 5. Effect of hydroxyl radical modulators on cellular ROS and hydroxyl radical levels. H460, H460/Cav-1, and H460/shCav-1 cells were treated with 200 μM H₂O₂ in the presence of deferoxamine (DFO, 1 mM) (a and c) or ferrous sulfate (50 μM) (b and d) and cellular ROS and hydroxyl radical levels were detected using DCFH₂-DA (a and b) and HPF probes (c and d), respectively. Data are the mean±S.D. (n=3).


