Dependence of Reactive Oxygen Species and FLICE Inhibitory Protein on Lipofectamine-Induced Apoptosis in Human Lung Epithelial Cells

Lalana Kongkaneramit, Narong Sarisuta, Neelam Azad, Yongju Lu, Anand Krishnan V. Iyer, Liying Wang, and Yon Rojanasakul

Department of Pharmaceutical Sciences, West Virginia University, Morgantown, West Virginia (L.K., N.A., Y.L., A.K.V.I., Y.R.); Department of Industrial Pharmacy, Mahidol University, Bangkok, Thailand (L.K., N.S.); and Pathology and Physiology Research Branch, National Institute for Occupational Safety and Health, Morgantown, West Virginia (L.W.)

ABSTRACT

Cationic liposomes such as Lipofectamine (LF) are widely used as nonviral gene delivery vectors; however, their clinical application is limited by their cytotoxicity. These agents have been shown to induce apoptosis as the primary mode of cell death, but their mechanism of action is not well understood. The present study investigated the mechanism of LF-induced apoptosis and examined the role of reactive oxygen species (ROS) in this process. We found that LF induced apoptosis of human epithelial H460 cells through a mechanism that involves caspase activation and ROS generation. Inhibition of caspase activity by pan-caspase inhibitor (z-VAD-fmk) or by specific caspase-8 inhibitor (z-IETD-fmk) or caspase-9 inhibitor (z-LEHD-fmk) inhibited the apoptotic effect of LF. Overexpression of FLICE-inhibitory protein (FLIP) or B-cell lymphoma-2, which are known inhibitors of the extrinsic and intrinsic death pathways, respectively, similarly inhibited apoptosis induced by LF. Induction of apoptosis by LF was shown to require ROS generation because its inhibition by ROS scavengers or by ectopic expression of antioxidant enzyme superoxide dismutase and glutathione peroxidase strongly inhibited the apoptotic effect of LF. Electron spin resonance studies showed that LF induced multiple ROS; however, superoxide was found to be the primary ROS responsible for LF-induced apoptosis. The mechanism by which ROS mediate the apoptotic effect of LF involves down-regulation of FLIP through the ubiquitination pathway. In demonstrating the role of FLIP and ROS in LF death signaling, we document a novel mechanism of apoptosis regulation that may be exploited to decrease cytotoxicity and increase gene transfection efficiency of cationic liposomes.

Cationic liposomes are widely used for direct gene transfer to introduce DNA encoding therapeutic proteins. Compared with anionic or neutral liposomes, cationic liposomes are more widely used due to the stable complex formed between the positively charged lipoparticle and the negatively charged DNA. In addition, the overall positive charge of the complex interacts with the negative charge of the plasma membrane and facilitates internalization of the complex into the cells by endocytosis (Azad and Rojanasakul, 2006). These nonviral vector systems are considered as an attractive alternative to viral vectors due to their biocompatibility, versatility, ease of preparation, and scale-up (Li and Huang, 2006). However, a major drawback in the application of this system for clinical purposes is its cytotoxicity, which often limits the transfection efficiency and gene expression. Cytotoxicity of cationic liposomes is closely associated with the charge ratio between the cationic species and the nucleic acids as well as the dose of lipoplexes administered. Higher charge ratios are generally more toxic (Dokka et al., 2000; Lv et al., 2006), whereas neutral and anionic liposomes are less toxic (Aramaki et al., 2000; Dokka et al., 2000). Thus, cationic liposomes can only be used in limited quantities that consequently limit their transfection efficiency. Recent evidence also suggests that the cytotoxic effects of cationic liposomes include cell shrinking, reduced number of mitosis, vacuolization, and the formation of balloon cells.
tion of the cytoplasm, and cell death (Iwaoka et al., 2006). It is well established that cell death induced by cationic liposomes occurs primarily through apoptosis (Aramaki et al., 1999, 2000).

Apoptosis is a normal physiological process that leads to cell death mediated by programmed signaling pathways (Hengartner, 2000; Cho and Choi, 2002). Caspases, a family of cysteine proteases, are important signaling proteins that are activated specifically in apoptotic cells (Wolf and Green, 1999; Hengartner, 2000). Two major pathways of apoptosis that lead to the activation of caspases, viz., death receptor and mitochondrial pathway, have been recognized (Ashkenazi and Dixit, 1998; Green and Reed, 1998; Fan et al., 2005). Death receptor pathway is initiated by cell death signals that lead to the binding of death ligands to the death receptors including Fas (CD95/APO-1) and tumor necrosis factor receptor-1 located in the plasma membrane. This initiates binding of specialized adaptor proteins such as Fas-associated death domain (FADD) to their cytosolic death domains, resulting in the recruitment of procaspase-8 to the death-inducing signaling complex, causing activation of caspase-8, which in turn activates effector caspses such as caspase-3 (Irmler et al., 1997; Wallach et al., 1999). In contrast to the death receptor pathway, the mitochondrial death pathway can be induced by the translocation of proapoptotic proteins such as Bid, Bax, and Bad from the cytosol to the mitochondria, where they promote cytochrome c release. Cytochrome c then binds to apoptotic protease activating factor-1 and forms an activation complex (apoptosome) with procaspase-9, leading to its cleavage and activation in the form of caspase-9 (Green and Reed, 1998) that activates effector caspases leading to apoptosis. Antiapoptotic regulatory proteins such as FLICE-inhibitory protein (FLIP) and B-cell lymphoma-2 (Bcl-2) can modulate the activation process of caspase-8 and caspase-9, respectively (Budihardjo et al., 1999). Upon stimulation, FLIP interferes with procaspase-8 binding to FADD at the death-inducing signaling complex, potently inhibiting apoptosis induced via the death receptor pathway (Irmler et al., 1997). Bcl-2 can prevent apoptosis via the mitochondrial pathway by inhibiting the release of cytochrome c from the mitochondria (Yang et al., 1997).

Several reports have established that induction of apoptosis by a variety of agents is closely associated with cellular oxidative stress (Pierce et al., 1991; Simon et al., 2000; Alexandre et al., 2006). Another report suggested that reactive oxygen species (ROS) acts upstream of p38/mitogen-activated protein kinase and plays a key role in tumor necrosis factor-related apoptosis-inducing ligand/Apo2L-induced apoptosis (Lee et al., 2002). Furthermore, Moungjaroen et al. (2006) demonstrated that ROS can mediate caspase activation and apoptosis through Bcl-2 down-regulation. Likewise, ROS have been shown to play a key role in cationic liposome-mediated cytotoxicity (Dokka et al., 2000). This effect was charge-dependent and can be prevented by ROS scavengers. Studies on cationic liposome-induced apoptosis also showed that antioxidants can inhibit apoptosis induced by stearylamine liposome (Aramaki et al., 1999, 2000), suggesting the important role of ROS in the induction of apoptosis by cationic liposomes. However, exactly how ROS mediates the apoptotic effect of cationic liposomes is unclear. Furthermore, the identity of specific ROS involved in the apoptotic process has not been investigated. The present study was undertaken to determine the role of specific ROS in Lipofectamine (LF)-induced apoptosis and elucidate the underlying mechanism.

Materials and Methods

Chemicals and Reagents. Lipofectamine was obtained from Invitrogen (Carlsbad, CA). This liposomal formulation is composed of a 3:1 mixture of cationic lipid DOSPA and neutral lipid DOPE. Fluoresgenic caspase substrates (CaspGLOW fluorescein active caspase-8 and caspase-9), pan-caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk), and caspase-9 inhibitor (z-LEHD-fmk) were obtained from BioVision (Mountain View, CA). Cell-permeable superoxide dismutase (SOD) mimetic MnTMPyP was obtained from Calbiochem (La Jolla, CA), and catalase (CAT) was obtained from Roche Diagnostics (Indianapolis, IN). The spin trapping agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), fluorescent ROS probes dihydroethidium (DHE) and dichlorofluorescein diacetate (DCF-DA), Hoechst 33342, diphenylene iodonium (DPI), and rotenone were obtained from Sigma Chemical (St. Louis, MO). Monoclonal antibody against FLIP (Dive-2) was obtained from Alexis Biochemical (San Diego, CA). Peroxidase-conjugated anti-Myc antibody (9E10), anti-Myc agarose beads, and protein A-agarose were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for ubiquitin, β-actin, and peroxidase-conjugated secondary antibodies were obtained from Sigma Chemical.

Cell Culture. Human lung epithelial NCI-H460 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin. The cells were grown in a humidified atmosphere of 5% CO2 at 37°C until they reached approximately 80% confluence before use. In all experiments, cells were treated with LF in the absence of DNA, with the exception of gene transfection studies described below.

Apoptosis and Caspase Activity Assays. Apoptosis was determined by Hoechst 33342 and DNA ladder assays. For Hoechst assay, cells were incubated with 10 μg/ml of the Hoechst dye at 37°C for 30 min and analyzed for apoptosis by scoring the percentage of cells having intensely condensed chromatin and/or fragmented nuclei by fluorescence microscopy (Leica, Wetzlar, Germany). Approximately 1000 nuclei from random fields were analyzed for each sample. The apoptotic index was calculated as apoptotic nuclei/total nuclei × 100 (%). For DNA ladder assay, cells were lysed with a lysis buffer (5 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 0.5% Triton X-100) on ice for 45 min and centrifuged at 14,000g for 30 min at 4°C. DNA in the supernatant was extracted twice with phenol/chloroform/isomyl-alcohol (25:24:1, v/v/v) and once with chloroform, then precipitated with ethanol and salt. The DNA pellet was washed once with 70% ethanol and resuspended in TE buffer, pH 8.0, containing 100 μg/ml RNase at 37°C for 2 h. The DNA fragments were separated by gel electrophoresis at 20 V for 18 h through a 2% Tris borate EDTA agarose gel containing 1 μg/ml ethidium bromide. The separated DNA fragments were examined under a UV transilluminator and photographed.

Caspase activity was determined by fluorometry using CaspGLOW caspase-8 and caspase-9 assay kits from BioVision, according to the manufacturer’s instructions. Caspase-specific fluorescent substrates (CaspGLOW fluorescein active caspase-8 and caspase-9), pan-caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk), and caspase-9 inhibitor (z-LEHD-fmk) were obtained from BioVision (Mountain View, CA). The cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and peroxidase-conjugated secondary antibodies were obtained from Sigma Chemical.

Electron spin resonance (ESR) was used to determine short-lived free radical generation with the aid of the spin trapping agent DMPO. The intensity of the spin adduct signal was used to measure the amount of short-lived radicals trapped, and the hyperfine couplings of the spin adduct were generally characteristics
of the original trapped radicals. All ESR measurements were carried out using a Varian E9 ESR spectrometer and a flat cell assembly (Varian Inc., Palo Alto, CA). Reactants were mixed in a test tube in a final volume of 0.5 ml at 37°C. The reaction mixture was then transferred to a flat cell for measurement. Hyperfine couplings were measured (0.1 G) directly from magnetic field separation using potassium tetraperoxochromate and 1,1-diphenyl-2-picrylhydrazyl as reference standards. The software EPRDAP, version 2.0, was used for data acquisition and analysis.

Fluorometric analysis of superoxide and peroxide formation was performed using DHE and DCF-DA as fluorescent probes. Cells were incubated with the probes (10 µM) at 37°C for 30 min, after which they were washed, resuspended in phosphate-buffered saline, and immediately analyzed for fluorescence intensity using a multiwell plate reader (FLUOstar OPTIMA; BMG Labtech Inc.) at the excitation/emission wavelengths of 485/610 nm for DHE measurements and 485/530 nm for DCF measurements.

**Plasmids and Stable Transfection.** FLIP, SOD, and glutathione peroxidase (Gpx) plasmids were generously provided by Dr. Christian Stehlik (Northwestern University, Chicago, IL). Authenticity of the plasmid constructs was verified by DNA sequencing. Stable transfectants were generated by culturing H460 cells in a 6-well plate until they reached 80% confluence. Ten hours later, the medium was replaced with culture medium containing 5% fetal bovine serum, and approximately 36 h after the beginning of the transfection, the cells were digested with 0.03% trypsin and the cell suspensions were plated onto 75-cm² culture flasks. The cells were cultured for 4 weeks with G418 selection (400 µg/ml). Resistant transfectants were isolated using cloning cylinders (Bellco Glass, Vineland, NJ) and transferred for expansion and analysis by Western blotting. Stable transfectants were grown in G418-free RPMI medium for at least two passages before each experiment.

**Western Blotting.** Cell extracts were performed by incubating the cells in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture for 30 min on ice. After insoluble debris was pelleted by centrifugation at 14,000 g for 15 min at 4°C, the supernatants were collected and analyzed for protein content using bicinchoninic acid assay. Proteins (20 µg) were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis DNA ladder assays. Hoechst assay shows that treatment of the cells with LF (0–50 µg/ml) caused a dose- and time-dependent increase in apoptosis over control level (Fig. 1, A and B). Higher doses of LF (>50 µg/ml) decreased apoptotic cell death possibly due to necrosis as

### Results

**Lipofectamine Induces Apoptosis of Human Lung Epithelial H460 Cells.** Cationic liposomes have been shown to induce apoptosis as the primary mode of cell death in various cell types (Aramaki et al., 1999, 2000). To test whether the cationic liposome LF could induce apoptosis in human lung epithelial cells and to determine the underlying mechanism, we first characterized the apoptotic response to LF treatment in H460 cells using Hoechst 33342 and gel electrophoresis DNA ladder assays. Hoechst assay shows that treatment of the cells with LF (0–50 µg/ml) caused a dose- and time-dependent increase in apoptosis over control level (Fig. 1, A and B). Higher doses of LF (>50 µg/ml) decreased apoptotic cell death possibly due to necrosis as

**Immunoprecipitation.** Cells were washed after treatments with ice-cold phosphate-buffered saline and incubated in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 0.2% NP40, 100 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture) for 20 min at 4°C. After centrifugation at 14,000g for 15 min at 4°C, the supernatants were collected and analyzed for protein content using bicinchoninic acid assay. Proteins (20 µg) were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and transferred onto 0.45-µm nitrocellulose membranes. The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.05% Tween-20) and incubated with appropriate primary antibodies at 4°C overnight. Membranes were washed three times with TBST for 10 min and incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immune complexes were detected by chemiluminescence (SuperSignal West Pico; Pierce Biotechnology, Rockford, IL) and quantified by imaging densitometry using UN-SCAN-IT automated digitizing software (Silk Scientific Inc., Orem, UT). Mean densitometry data from independent experiments were normalized to the control.

**Fig. 1.** Lipofectamine induces apoptosis of human lung epithelial H460 cells. A, subconfluent (80%) monolayers of H460 cells were treated with varying concentrations of LF (0–50 µg/ml), and apoptosis was determined by Hoechst 33342 assay after 6 h. B, cell monolayers were treated with LF (20 µg/ml) for various times, and apoptosis was similarly determined. C, representative fluorescence micrographs of cells treated with LF (0, 20, and 40 µg/ml) for 6 h and stained with the Hoechst dye. Apoptotic cells exhibit condensed nuclei with bright nuclear fluorescence (original magnification, 400×). D, cells were treated with varying doses of LF (0–50 µg/ml) and analyzed for DNA fragmentation by gel electrophoresis DNA ladder assay after 6 h. Data are mean ± S.D. (n = 4). *, p < 0.05 versus nontreated control.
indicated by propidium iodide assay (data not shown). Apoptotic cells exhibited condensed nuclei with intense nuclear fluorescence (Fig. 1C). DNA ladder assay that measures DNA fragmentation, a hallmark of apoptosis, also shows that LF induced a dose-dependent increase in fragmented DNA levels (Fig. 1D), consistent with the apoptosis assay by Hoechst nuclear fluorescence.

**Apoptosis Pathways and Caspase Activation by Lipofectamine.** To determine the apoptosis pathway and requirement of caspases in the induction of apoptosis by LF, we analyzed caspase-8 and caspase-9 activities in LF-treated cells. Figure 2A shows that both caspase-8 and -9 were activated by the LF treatment in a dose-dependent manner. Because caspase-8 serves as the apical caspase of the extrinsic (death receptor) pathway whereas caspase-9 represents the apical caspase of the intrinsic (mitochondrial) pathway (Green and Reed, 1998; Wallach et al., 1999), these results suggest that the two pathways are involved in the induction of apoptosis by LF. Control experiments using specific caspase-8 inhibitor (z-IETD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) showed a significant reduction in apoptosis induced by LF, whereas the pan-caspase inhibitor z-VAD-fmk almost completely inhibited the apoptotic effect of LF (Fig. 2B). These results indicate that apoptosis induced by LF is mediated through the classic caspase-dependent pathways.

To confirm the above finding, cells were stably transfected with FLIP, a known inhibitor of the death receptor pathway (Irmler et al., 1997; Chang et al., 2002), and Bcl-2, an inhibitor of the mitochondrial death pathway (Sun et al., 2002; Chanvorachote et al., 2006), and their effect on LF-induced apoptosis was examined. FLIP and Bcl-2 gene transfection resulted in a corresponding increase in the protein expression levels over vector-transfected control, as determined by Western blot analysis (Fig. 3A). Treatment of the vector-transfected cells with LF caused apoptosis similar to that observed in nontransfected cells, whereas the same treatment produced a significantly lower level of apoptosis in FLIP-overexpressing cells (Fig. 3B). FLIP overexpression also inhibited LF-induced caspase-8 and -9 activation (Fig. 3, C and D), suggesting the linkage of the two death pathways, i.e., via Bid cleavage, which has previously been shown by other groups using cationic stearylamine liposome (Aramaki et al., 2000; Iwaoka et al., 2006). Apoptosis was also reduced in Bcl-2-overexpressing cells in response to LF treatment; however, such reduction was less pronounced than that observed in FLIP-overexpressing cells (Fig. 3B). Analysis of caspase-8 and caspase-9 activities in Bcl-2-overexpressing cells shows that the increase in Bcl-2 expression had no significant effect on LF-induced caspase-8 activation (Fig. 3D).
3C) but had a partial inhibitory effect on caspase-9 activation (Fig. 3D). These results suggest that the antiapoptotic effect of Bel-2 was mediated downstream of the caspase-8 activation pathway.

**Lipofectamine Down-Regulates FLIP through an ROS-Dependent Mechanism.** ROS has been shown to mediate the cytotoxic effect of cationic liposomes (Dokka et al., 2000). To test whether ROS might mediate the apoptotic effect of LF through FLIP, which was shown to play a key role in the apoptotic process, we analyzed FLIP protein expression in response to LF treatment in the presence and absence of various known ROS inhibitors. Figure 4A shows that treatment of the cells with LF caused a dose-dependent decrease in the expression level of FLIP as determined by Western blotting. A similar LF treatment had no significant effect on the expression levels of FADD and Fas, which are known to be involved in the extrinsic death pathway. Pretreatment of the cells with SOD mimetic MnTBAP (O$_2^-$ scavenger) completely inhibited the FLIP down-regulation, whereas pretreatment of the cells with CAT (H$_2$O$_2$ scavenger) and sodium formate (OH$^-$ scavenger) had partial and no inhibitory effect, respectively, on FLIP down-regulation (Fig. 4B). These results suggest that O$_2^-$ and to a lesser extent H$_2$O$_2$, plays an important role in the regulation of FLIP and death signaling induced by LF.

**Lipofectamine Induces Multiple ROS Generation.** To provide a relationship between cellular FLIP and ROS responses to LF treatment, we performed ESR studies analyzing ROS generation using the spin trap DMPO. The ESR technique was used because it allows identification of the specific ROS involved. Cells were treated with LF in the presence or absence of specific ROS scavengers and were analyzed for ROS generation. Nontreated cells with DMPO were used as a negative control. Figure 5A shows that in the

---

**Fig. 4.** Lipofectamine induces down-regulation of FLIP and its inhibition by antioxidants. A, subconfluent (80%) monolayers of H460 cells were treated with varying concentrations of LF (0–40 µg/ml) for 6 h, and cell lysates (50 µg of protein) were prepared and analyzed for FLIP, FADD, Fas, and Fas protein expression by Western blotting. Blots were also probed with β-actin antibody to confirm equal loading of the samples. Immunoblot signals were quantified by densitometry, and mean data from independent experiments (one of which is shown here) were normalized to the result obtained in nontreated cells (control). B, cells were either left untreated or pretreated with MnTBAP (100 µM), CAT (1000 units/ml), or sodium formate (10 mM) for 1 h, and then treated with LF (20 µg/ml) for 6 h. Cell lysates were prepared and analyzed for FLIP by Western blotting. Plots are mean ± S.D. (n = 3). *, p < 0.05 versus nontreated control; **, p < 0.05 versus LF-treated control.

**Fig. 5.** Lipofectamine induces ROS generation and its inhibition by antioxidants. A, H460 cells were grown in culture until they reached 80% confluency, and cell lysates (50 µg of protein) were prepared and analyzed for FLIP, FADD, and Fas protein expression by Western blotting. Blots were also probed with β-actin antibody to confirm equal loading of the samples. Immunoblot signals were quantified by densitometry, and mean data from independent experiments (one of which is shown here) were normalized to the result obtained in nontreated cells (control). B, cells were either left untreated or pretreated with MnTBAP (100 µM) for 1 h, and then treated with LF (20 µg/ml) for 6 h. Cell lysates were prepared and analyzed for FLIP by Western blotting. Plots are mean ± S.D. (n = 3). *, p < 0.05 versus nontreated control; **, p < 0.05 versus LF-treated control.
absence of LF, no ESR signal was observed. However, in the presence of added LF, a clear signal consisting of a 1:2:2:1 quartet was detected. Based on line shape and hyperfine splitting of the spectrum, the signal was assigned to the DMPO-OH\(^{•}\) adduct, which is indicative of OH\(^{•}\) generation. The formation of DMPO-OH\(^{•}\) adduct was detected as early as 5 min and peaked at approximately 40 min after the treatment, where it gradually declined to the baseline level (data not shown).

Addition of the OH\(^{•}\) scavenger sodium formate strongly inhibited the ESR signal, indicating the specificity of OH\(^{•}\) detection. Addition of MnTBAP or CAT also inhibited the signal intensity, indicating that O\(_2\)\(^{•}\) and H\(_2\)O\(_2\) were generated in LF-treated cells, and that these oxidative species were precursors for OH\(^{•}\) generation. The formation of O\(_2\)\(^{•}\) and H\(_2\)O\(_2\) in the treated cells was confirmed by spectrofluorometry using fluorescent probes DHE and DCF-DA, respectively (Fig. 5B). To determine the cellular source of ROS generation induced by LF, cells were treated with LF in the presence or absence of DPI, a specific inhibitor of NADPH oxidase (Irani et al., 1997; Moungjaroen et al., 2006), or rotenone, a mitochondrial electron transport chain interrupter (Irani et al., 1997; Chen et al., 2003), and their effect on ROS generation was examined by DHE fluorometry. The results show that DPI strongly inhibited LF-induced DHE fluorescence, whereas rotenone showed minimal effect (Fig. 5C). These results suggest that the plasma membrane NADPH oxidase is a key source of superoxide generation induced by LF in the treated cells.

Overexpression of Antioxidant Enzymes Inhibits Lipofectamine-Induced Apoptosis and FLIP Down-Regulation. To confirm the role of ROS in FLIP down-regulation and to test the effect of ROS on LF-induced apoptosis, cells were stably transfected with the antioxidant enzyme SOD, GPx, or control plasmid, and their effects on FLIP expression, ROS generation, apoptosis, and caspase activation by LF were examined. Transfection of the cells with SOD and GPx resulted in a corresponding increase in the antioxidant enzyme expression levels compared with vector-transfected control (Fig. 6A). Overexpression of SOD potently inhibited LF-induced FLIP down-regulation (Fig. 6B) and O\(_2\)\(^{•}\) generation (Fig. 6C) compared with vector-transfected control. Such overexpression also inhibited apoptosis (Fig. 7A) and caspase-8 and -9 activation (Fig. 7, B and C) induced by LF. Consistent with our earlier catalase studies, overexpression of GPx showed less inhibitory effects on LF-induced FLIP down-regulation, O\(_2\)\(^{•}\) generation, apoptosis, and caspase activation. Together, these results suggest that although H\(_2\)O\(_2\) is involved in LF-induced apoptosis, O\(_2\)\(^{•}\) is the major regulator of cell death and FLIP down-regulation induced by LF.

Down-Regulation of FLIP Is Mediated through ROS-Dependent Ubiquitination. FLIP has been shown to be down-regulated by ubiquitination and proteasomal degradation in various apoptosis conditions (Kim et al., 2002; Perez and White, 2003; Chanvorachote et al., 2005). To determine whether this regulatory pathway is involved in the down-regulation of FLIP by LF and whether ROS plays a role in this process, we performed immunoprecipitation studies analyzing FLIP ubiquitination in cells treated with LF in the presence or absence of antioxidants. Cells were transiently transfected with ubiquitin and myc-tagged FLIP plasmids, and the resulting immune complexes were analyzed by SDS-PAGE immunoblotting using anti-ubiquitin antibody. Figure 8A shows that in the absence of LF, minimal ubiquitinated FLIP was produced. Upon LF treatment, the level of ubiquitinated FLIP was greatly increased. Pretreatment of the cells with MnTBAP strongly inhibited the ubiquitination of FLIP, whereas CAT was less effective. These results indicate the role of O\(_2\)\(^{•}\) as the major mediator of FLIP ubiquitination, consistent with its role in LF-induced FLIP down-regulation (Fig. 4B). We also performed apoptosis studies in cells treated with LF in the presence and absence of MnTBAP and CAT (Fig. 8B). The results show that at the same treatment doses, MnTBAP strongly inhibited the apoptotic effect of LF, whereas catalase showed less inhibitory effect. These results are in good agreement with our earlier antioxidant enzyme overexpression studies and support the role of ROS, particularly O\(_2\)\(^{•}\) in apoptosis and FLIP down-regulation by LF.

Discussion

Cationic liposomes have been successfully used in gene transfection and are considered as promising tools for gene delivery. However, limited understanding of their cytotoxicity and the underlying mechanism is the major drawback in the successful application of these agents. LF is one of the most widely used cationic liposomal agents due to its high transfection efficiency compared with several other transfecting agents such as Lipofectin, DEAE-dextran, and DOTAP.
is treated with LF (20 μg/ml) for 6 h and analyzed for apoptosis by Hoechst assay. B and C, transfected cells were similarly treated with LF and analyzed for caspase-8 and -9 activities by fluorometric caspase assays. A, H460 cells were stably transfected with SOD, GPx, or control plasmid. Transfected cells were treated with LF (20 μg/ml) for 6 h and analyzed for apoptosis by Hoechst assay. B and C, transfected cells were similarly treated with LF and analyzed for caspase-8 and -9 activities by fluorometric caspase assays. Plots are mean ± S.D. (n = 4). *, p < 0.05 versus nontreated control.

Fig. 7. Effects of antioxidant enzyme overexpression on Lipofectamine-induced apoptosis and caspase activation. A, H460 cells were stably transfected with SOD, GPx, or control plasmid. Transfected cells were treated with LF (20 μg/ml) for 6 h and analyzed for apoptosis by Hoechst assay. B and C, transfected cells were similarly treated with LF and analyzed for caspase-8 and -9 activities by fluorometric caspase assays. Plots are mean ± S.D. (n = 4). *, p < 0.05 versus nontreated control.

Fig. 8. Effects of Lipofectamine and antioxidants on FLIP ubiquitination and apoptosis. A, H460 cells were transiently transfected with ubiquitin and myc-tagged FLIP plasmids. Thirty-six hours later, the cells were treated with LF (20 μg/ml) in the presence or absence of MnTBAP (100 μM) or catalase (1000 units/ml). Cell lysates were immunoprecipitated with anti-myc antibody, and the immune complexes were analyzed for ubiquitin by Western blotting. Analysis of ubiquitin was performed at 2 h post-treatment, where ubiquitination was found to be maximal. B, H460 cells were treated with LF with or without MnTBAP or catalase as described, and they were analyzed for apoptosis after 6 h. Data are mean ± S.D. (n = 3). *, p < 0.05 versus nontreated control; **, p < 0.05 versus LF-treated control.

complexes has been attributed to the excess charges of cationic liposome, the results of this study should be useful in designing a safer and perhaps more effective gene delivery system.

We also studied the mechanism by which ROS mediates the apoptotic effect of LF. We found that LF activates both the intrinsic (mitochondrial) and extrinsic (death receptor) pathways of apoptosis (Fig. 2). The induction of apoptosis through the death receptor pathway involves activation of caspase-8, which is inhibited by the addition of caspase-8-specific inhibitor (z-IETD-fmk) or pan-caspase inhibitor (z-VAD-fmk) (Fig. 2). Moreover, overexpression of FLIP, a known inhibitor of the death receptor pathway, strongly inhibited the apoptotic effect of LF, whereas overexpression of Bcl-2, an inhibitor of the mitochondrial death pathway, or addition of caspase-9 inhibitor (z-LEDH-fmk) showed lesser effect (Figs. 2B and 3B). It is possible that the two death pathways activated by LF are linked. Previous studies by Aramaki et al. (1999, 2000) showed that stearylamine cationic liposome activated caspase-8 and induced Bid cleavage, which leads to its translocation into the mitochondria. Such translocation was further shown to promote cytochrome c release and subsequent activation of the mitochondrial death pathway (Iwaoka et al., 2006).
The mechanism by which cationic liposomes activate the death receptor pathway is unknown. Immunoblot analysis of key apoptosis-regulatory proteins involved in the death receptor pathway shows that only FLIP was down-regulated by the LF treatment, whereas FADD and Fas expression levels were relatively unchanged (Fig. 4A). Ectopic expression of FLIP decreased cell death induced by LF (Fig. 3B), supporting the antiauxotopic role of this protein, and suggesting that down-regulation of FLIP by LF is a key event in the death signaling. Overexpression of FLIP also inhibited caspase-8 activation by LF (Fig. 3C), supporting the above notion. The antiauxotopic function of FLIP is tightly associated with its expression levels, and down-regulation of FLIP is an important mechanism to sensitize cells to receptor-mediated apoptosis (Krueger et al., 2001).

Several apoptotic stimuli and conditions such as chemotherapy agents (Kim et al., 2002; Nitobe et al., 2003; Day et al., 2006), viral infection (Perez and White, 2003), and p53 and death receptor activation (Fukazawa et al., 2001; Chanvorachote et al., 2005), have been shown to induce FLIP down-regulation through the ubiquitination pathway. Consistent with these findings, we found that LF induced ubiquitination of FLIP (Fig. 8A), and that its inhibition by antioxidants blocked the down-regulation of FLIP by LF (Figs. 4B and 6B). The results of this study also indicate $O_2^-$ is the major ROS involved in FLIP ubiquitination because its inhibition by MnTBAP potently inhibited the ubiquitination compared with catalase (Fig. 8B). This finding is consistent with the apoptosis and FLIP expression data and indicate the dominant role of $O_2^-$ in LF death signaling. Because many apoptotic stimuli of the death receptor pathway are known to induce ROS generation, the results of this study also suggest that ROS may be a common mediator of FLIP ubiquitination and apoptosis induced by various apoptotic agents.

The mechanism by which ROS mediates the ubiquitination of FLIP remains to be further elucidated. ROS may exert its effect directly on the protein by interacting with specific amino acid residues leading to conformational changes and increased susceptibility of the protein to ubiquitination by ubiquitin ligases. FLIP has also been shown to be negatively regulated by nitric oxide (NO), which induces $S$-nitrosylation and inhibits ubiquitination of the protein (Chanvorachote et al., 2005). Because ROS such as $O_2^-$ is known to interact with NO in cellular systems (Burutia and Brown, 2003; Wenzel et al., 2003), and because $O_2^-$ was shown to be critical in FLIP ubiquitination by LF, it is possible that $O_2^-$ may promote FLIP ubiquitination through NO scavenging. Furthermore, ROS may affect FLIP by up-regulating or activating the ubiquitin ligase responsible for FLIP ubiquitination.

In summary, our data provide evidence that FLIP plays an important role in the regulation of apoptosis induced by LF in human lung epithelial H460 cells. Apoptosis induced by LF is mediated in part through the death receptor pathway via caspase-8 activation, which is negatively regulated by FLIP. LF induces rapid generation of ROS that is required for FLIP down-regulation and subsequent activation of apoptosis, because inhibition of ROS by antioxidants prevented these effects. LF induces down-regulation of FLIP through a process that involves ROS-mediated ubiquitination.

Superoxide plays an important role in this process as well as in apoptosis induced by the liposomal agent. This oxidative species may represent a common regulator of FLIP function that controls apoptotic cell death induced by various physiologic and pathologic stimuli. In showing the role of FLIP and its regulation by ROS, we document a novel mechanism of apoptosis induction by LF, which may be useful in increasing the efficiency of gene expression and allow more effective treatment of diseases by gene-based therapies.

References


Address correspondence to: Dr. Yon Rojanasakul, West Virginia University, Health Sciences Center, Department of Pharmaceutical Sciences, P.O. Box 9530, Morgantown, WV 26506. E-mail: yrojan@hsc.wvu.edu