The Fas Death Signaling Pathway Connecting Reactive Oxygen Species Generation and FLICE Inhibitory Protein Down-Regulation

Lijing Wang, Neelam Azad, Lalana Kongkaneramit, Fei Chen, Yongju Lu, Bing-Hua Jiang and Yon Rojanasakul

*J Immunol* 2008; 180:3072-3080; doi: 10.4049/jimmunol.180.5.3072

http://www.jimmunol.org/content/180/5/3072

---

**References**

This article cites 59 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/180/5/3072.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Fas Death Signaling Pathway Connecting Reactive Oxygen Species Generation and FLICE Inhibitory Protein Down-Regulation

Lifying Wang,* Neelam Azad,† Lalana Kongkaneramit,† Fei Chen,* Yongju Lu,† Bing-Hua Jiang,‡ and Yon Rojanasakul†‡

Fas-mediated apoptosis plays an important role in normal tissue homeostasis, and disruption of this death pathway contributes to many human diseases. Induction of apoptosis via Fas activation has been associated with reactive oxygen species (ROS) generation and down-regulation of FLICE inhibitory protein (FLIP); however, the relationship between these two events and their role in Fas-mediated apoptosis is unclear. We show herein that ROS are required for FLIP down-regulation and apoptosis induction by Fas ligand (FasL) in primary lung epithelial cells. ROS mediate the down-regulation of FLIP by ubiquitination and subsequent degradation by proteasome. Inhibition of ROS by antioxidants or by ectopic expression of ROS-scavenging enzymes glutathione peroxidase and superoxide dismutase effectively inhibited FLIP down-regulation and apoptosis induction by FasL. Hydrogen peroxide is a primary oxidative species responsible for FLIP down-regulation, whereas superoxide serves as a source of peroxide and a scavenger of NO, which positively regulates FLIP via S-nitrosylation. NADPH oxidase is a key source of ROS generation induced by FasL, and its inhibition by dominant-negative Rac1 expression or by chemical inhibitor decreased the cell death response to FasL. Taken together, our results indicate a novel pathway of FLIP regulation by an interactive network of reactive oxygen and nitrogen species that provides a key mechanism of apoptosis regulation in Fas-induced cell death and related apoptosis disorders. The Journal of Immunology, 2008, 180: 3072–3080.

Although Fas activation can trigger apoptotic cell death, the expression of Fas receptor does not necessarily render cells susceptible to death by its ligand (2, 5), indicating that inhibitors of the apoptosis signaling pathway exist and play a role in the apoptotic process. A key apoptosis regulatory protein of the Fas death pathway is FLICE inhibitory protein (FLIP). FLIP has been shown to inhibit apoptosis by preventing the binding of procaspase-8 to FADD or interfering with the autocatalytic activation of caspase-8 at the death-inducing signaling complex (14, 15). Although the antiapoptotic role of FLIP has been well documented (reviewed in Ref. 16), several studies have shown that FLIP may also act as an inducer of apoptosis depending on its expression level and cell type (17, 18). To date, the role of FLIP has been exclusively studied in transformed cell lines. Because these cell lines may exhibit defective apoptosis machinery, its role in normal cells remains unclear. In this study, we sought to determine the role of FLIP in normal lung epithelial cells, which are a known target for Fas-induced apoptosis (8, 9), and to elucidate its mechanisms of Fas death regulation.

Activation of Fas receptor has been shown to be associated with rapid generation of reactive oxygen species (ROS) (19–22). However, the significance of this finding and the role of specific ROS involved in the apoptotic process are unclear. Vercammen et al. (19) demonstrated a dual pathway of apoptotic and necrotic cell death resulting from massive ROS generation and caspase activation. Gulbins et al. (20) and Sato et al. (21) implicated superoxide anion (O2•−) as a functional mediator of Fas-induced apoptosis in Jurkat cells, whereas Medan et al. (22) reported hydrogen peroxide (H2O2) and hydroxyl radicals (OH•) as key mediators in macrophages. In contrast, Hug et al. (23) found no requirement of ROS in fibroblasts stably expressing human Fas. The present study was undertaken to further clarify the role of ROS in Fas-induced...
apoptosis and to determine the specific ROS involved, their cellular sources, and the mechanisms by which they regulate Fas apoptosis. We also tested the hypothesis that FLIP is a key cellular target of ROS that determines cell sensitivity to Fas-induced apoptosis. Using various molecular and cellular approaches, we found that FLIP plays a crucial role as a negative regulator of Fas-induced apoptosis in lung epithelial cells. FLIP is down-regulated by FasL via an ROS-dependent ubiquitin-proteasomal degradation pathway. Our data also suggest the interaction between reactive oxygen and nitrogen species, which cooperatively regulate FLIP and Fas signaling, thus revealing a novel mechanism of cell death regulation in death receptor-mediated apoptosis.

Materials and Methods

Reagents and Abs

Recombinant FasL (SuperFasLigand), mAb to FLIP (Dave-2), and caspase-8 inhibitor (Z-IETF-FMK) were obtained from ALEXIS Biochemicals. Abs for Fas, FADD, caspase-8, and peroxidase-labeled secondary Abs and protein A-agarose were obtained from Santa Cruz Biotechnology. Abs for ubiquitin, S-nitrosocysteine, and β-actin were from Sigma-Aldrich, as were N-acetyl cysteine, rotenone, diphenylene iodo-nium, lactacystin, 2-(4-carboxyphenyl)-tetramethyluridazoline-1-oxo-3-oxide, and superoxide dismutase. Catalase was from Roche Diagnostics. The ROS and NO probes, dihydroethidine, dichlorofluorescein diacetate, and diaminofluorescein diacetate were from Molecular Probes. The transfecting agents Lipofectamine and Oligofectamine were from Invitrogen.

Cell isolation and culture

Alveolar epithelial type II cells were isolated from male Sprague-Dawley rats (100–150 g; Jackson Laboratories) as previously described (24). The animals were handled according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85-23, 1985). The animals were sacrificed with pentobarbital sodium (150 mg/kg body wt, ip) and the lungs were removed. They were perfused with 0.9% NaCl to remove blood cells and lavaged with PBS solution to remove free alveolar macrophages. The lungs were then excised and filled with PBS containing elastase (40 U/ml, type I; Worthington Biochemical) and DNase (0.006%; Sigma-Aldrich) and incubated at 37°C for 20 min. After enzymatic digestion, the lungs were finely minced and the digestion was arrested by incubation for 5 min in PBS containing 25% FBS and 0.006% DNase. The crude cell extract was sequentially filtered through 160- and 45-μm screens and then centrifuged. The resulting cell pellet was spun on a sterile Percoll density gradient, and the type II cell band was collected and resuspended in 1:1 (v/v) P12 and Eagle’s modified essential medium for further use.

Alveolar epithelial A549 cells were obtained from American Type Culture Collection. They were grown in F-12K medium containing 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

siRNA preparation and transfection

siRNA oligonucleotides against FLIP (siFLIP) and its control sequences (siCONTROL) were synthesized and obtained from Dharmacon using their SMARTpool and sDESIGN technology. The SMARTpool siRNA contains four sets of oligonucleotides specific for the sequences 1093–1111, 1103–1120, 1206–1224, and 1455–1473 of the long isoform of FLIP (FLIP,long) gene (accession no. U97074). The oligonucleotides were annealed and suspended to a concentration of 10 μM. Alveolar epithelial type II (AE2) cells were seeded for transfection in a low serum culture medium (1% FBS) without antibiotics. The cells were transfected with siFLIP or siCONTROL oligonucleotides (100 nM) using Oligofectamine according to the manufacturer’s instructions. The cells were incubated with the oligonucleotide-liposome complexes for 6 h, and then the low serum medium was replaced with normal medium (10% FBS) without antibiotics and incubated for an additional 48 h before further analysis.

Plasmids and stable transfection

The plasmids used in this study were generously provided by Dr. Christian Stiehlik (Northwestern University, School of Medicine, Chicago, IL). Authenticity of all plasmid constructs was verified by DNA sequencing. Stable transformants of FLIPΔ2, superoxide dismutase I (SOD1), glutathione peroxidase (GPx5), and dominant-negative Rac1 (Rac1N17) were generated by culturing A549 cells in a 6-well plate until they reached 80–90% confluence and to determine the specific ROS involved, their cellular sources, and the mechanisms by which they regulate Fas apoptosis. We also tested the hypothesis that FLIP is a key cellular target of ROS that determines cell sensitivity to Fas-induced apoptosis. Using various molecular and cellular approaches, we found that FLIP plays a crucial role as a negative regulator of Fas-induced apoptosis in lung epithelial cells. FLIP is down-regulated by FasL via an ROS-dependent ubiquitin-proteasomal degradation pathway. Our data also suggest the interaction between reactive oxygen and nitrogen species, which cooperatively regulate FLIP and Fas signaling, thus revealing a novel mechanism of cell death regulation in death receptor-mediated apoptosis.

Materials and Methods

Reagents and Abs

Recombinant FasL (SuperFasLigand), mAb to FLIP (Dave-2), and caspase-8 inhibitor (Z-IETF-FMK) were obtained from ALEXIS Biochemicals. Abs for Fas, FADD, caspase-8, and peroxidase-labeled secondary Abs and protein A-agarose were obtained from Santa Cruz Biotechnology. Abs for ubiquitin, S-nitrosocysteine, and β-actin were from Sigma-Aldrich, as were N-acetyl cysteine, rotenone, diphenylene iodo-nium, lactacystin, 2-(4-carboxyphenyl)-tetramethyluridazoline-1-oxo-3-oxide, and superoxide dismutase. Catalase was from Roche Diagnostics. The ROS and NO probes, dihydroethidine, dichlorofluorescein diacetate, and diaminofluorescein diacetate were from Molecular Probes. The transfecting agents Lipofectamine and Oligofectamine were from Invitrogen.

Cell isolation and culture

Alveolar epithelial type II cells were isolated from male Sprague-Dawley rats (100–150 g; Jackson Laboratories) as previously described (24). The animals were handled according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85-23, 1985). The animals were sacrificed with pentobarbital sodium (150 mg/kg body wt, ip) and the lungs were removed. They were perfused with 0.9% NaCl to remove blood cells and lavaged with PBS solution to remove free alveolar macrophages. The lungs were then excised and filled with PBS containing elastase (40 U/ml, type I; Worthington Biochemical) and DNase (0.006%; Sigma-Aldrich) and incubated at 37°C for 20 min. After enzymatic digestion, the lungs were finely minced and the digestion was arrested by incubation for 5 min in PBS containing 25% FBS and 0.006% DNase. The crude cell extract was sequentially filtered through 160- and 45-μm screens and then centrifuged. The resulting cell pellet was spun on a sterile Percoll density gradient, and the type II cell band was collected and resuspended in 1:1 (v/v) P12 and Eagle’s modified essential medium for further use.

Alveolar epithelial A549 cells were obtained from American Type Culture Collection. They were grown in F-12K medium containing 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

siRNA oligonucleotides against FLIP (siFLIP) and its control sequences (siCONTROL) were synthesized and obtained from Dharmacon using their custom SMARTpool and sDESIGN technology. The SMARTpool siRNA contains four sets of oligonucleotides specific for the sequences 1093–1111, 1103–1120, 1206–1224, and 1455–1473 of the long isoform of FLIP (FLIP,long) gene (accession no. U97074). The oligonucleotides were annealed and suspended to a concentration of 10 μM. Alveolar epithelial type II (AE2) cells were seeded for transfection in a low serum culture medium (1% FBS) without antibiotics. The cells were transfected with siFLIP or siCONTROL oligonucleotides (100 nM) using Oligofectamine according to the manufacturer’s instructions. The cells were incubated with the oligonucleotide-liposome complexes for 6 h, and then the low serum medium was replaced with normal medium (10% FBS) without antibiotics and incubated for an additional 48 h before further analysis.

Plasmids and stable transfection

The plasmids used in this study were generously provided by Dr. Christian Stiehlik (Northwestern University, School of Medicine, Chicago, IL). Authenticity of all plasmid constructs was verified by DNA sequencing. Stable transformants of FLIPΔ2, superoxide dismutase I (SOD1), glutathione peroxidase (GPx5), and dominant-negative Rac1 (Rac1N17) were generated by culturing A549 cells in a 6-well plate until they reached 80–90% con

FIGURE 1. FasL-induced apoptosis and caspase activation in lung epithelial cells. A. Primary AE2 cells (1 × 10⁶ cells/ml) were treated with varying concentrations of FasL (0–100 ng/ml) for 12 h, and apoptosis was determined by Hoechst 33342 assay. B. Fluorescence micrographs of cells treated with FasL (100 ng/ml) for 12 h. Apoptotic cells exhibited shrunken and fragmented nuclei with bright nuclear fluorescence. C. Cells were treated with FasL, as described in A and analyzed for DNA nucleosomal fragmentation by ELISA. D. Western blot analysis of caspase-8 at 6 h after FasL treatment. The blots were reprobed with an Ab specific for β-actin as a loading control. E. Cells were treated with FasL (100 ng/ml) in the presence or absence of caspase-8 inhibitor Z-IETF-FMK (10 μM) or NAC (100 μM), and apoptosis was determined by Hoechst assay at 12 h post-treatment. F. Cells were similarly treated as in E for 12 h, and cell lysates (50 μg protein) were prepared and analyzed for caspase-8 activity as described in Materials and Methods. Plots are means ± SD (n = 4), * p < 0.05 vs nontreated control. # p < 0.05 vs FasL-treated control.

ROS and NO detection

Cellular superoxide and peroxide formation were determined by flow cytometry using dihydroethidine (DHE) and 2',7'-dichlorofluorescein diacetate (DCF-DA) as fluorescent probes, respectively. For detection of NO, 4,5-diaminofluorescein diacetate (DAF-DA) was used as a probe. Cells were incubated with the probes (10 μM) for 30 min at 37°C in the dark. The cells were washed, resuspended in PBS, and analyzed for fluorescence intensity using FACSCalibur (BD Biosciences) at the excitation/emission wave-lengths of 488/610 nm for DHE and 488/538 nm for DCF and DAF fluorescence measurements. The median fluorescence intensity was quantified by CellQuest (BD Biosciences) software analysis of the recorded histograms.
Apoptosis and caspase assays

Apoptosis was determined by Hoechst 33342 assay and by ELISA-based DNA fragmentation assay using a kit from Roche Molecular Biochemicals. For Hoechst assay, cells were incubated with 10 ng/ml Hoechst 33342 for 30 min, and the percentage of cells having intensely condensed chromatin and/or fragmented nuclei by fluorescence microscopy (Carl Zeiss Axiovert) were scored using Pixera software. Approximately 1000 nuclei from random fields were analyzed for each sample. The apoptotic index was calculated as (apoptotic nuclei/total nuclei) x 100 (%). For ELISA, cells were lysed with DNA lysis buffer (200 µl), and the cell lysate (20 µl) was mixed with an Ab solution provided by the supplier (80 µl) in 96-well plates at room temperature for 2 h. After washing with the incubation buffer, the substrate buffer (100 µl) was added to each well and incubated for 10 min at 37°C. OD was then measured using a microplate reader at a wavelength of 405 nm.

Caspase-8 activity was determined by fluorometric assay using the enzyme substrate IETD-AMC (Alexis Biochemicals), which is specifically cleaved by the enzymes at the Asp residue to release the fluorescent leaving group, 7-amido-4-methyl coumarin (AMC). Cell extracts containing 20 µg protein were incubated with 100 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid containing 10% sucrose, 10 mM DTT, 0.1% CHAPS, and 50 µM caspase substrate in a total reaction volume of 250 µl. The reaction mixture was incubated for 2 h at 37°C. At the end of incubation, the liberated fluorescent group AMC was determined fluorometrically (RF-531PC spectrofluorometer, Shimadzu) at the excitation and emission wavelengths of 380 and 460 nm, respectively.

Western blotting

Cells were harvested, washed twice with cold PBS, and lysed in cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM NaF, 1 mM EGTA, 1 mM PMSF, 1 mM sodium orthovanadate, and 1% protease inhibitor (Roche Molecular Biochemicals) for 30 min. After cellular debris was precipitated by centrifugation at 14,000 g for 15 min at 4°C, the supernatants were collected and analyzed for protein content by bicinchoninic acid assay. Equal amount of proteins per sample (20 µg) were resolved on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. The transferred membrane was blocked for 1 h in 5% nonfat dry milk in TBST and incubated at 4°C overnight with primary Abs, including rat monoclonal anti-human c-FLIP Ab (804127, 1:500, ALEXIS Biochemicals), rabbit polyclonal anti-human Fas Ab (C20, 1:500, Santa Cruz Biotechnology), mouse monoclonal anti-ubiquitin Ab (U0508, 1:1000, Dako). 

FIGURE 2. Effects of antioxidants on FasL-induced apoptosis and ROS generation. A, AE2 cells (1 x 10^6 cells/ml) were either left untreated or pretreated for 1 h with varying concentrations of SOD (500, 1000, 5000 U/ml), catalase (500, 1000, 5000 U/ml), or sodium formate (5, 10, 20 mM). The cells were then treated with FasL (100 ng/ml) for 12 h and analyzed for apoptosis by Hoechst assay. B, Cells were treated with FasL (100 ng/ml) in the presence or absence of SOD (1000 U/ml), catalase (1000 U/ml), or sodium formate (10 mM), and they were analyzed for ROS production by measuring DHE and DCF fluorescence intensities. Plots show relative fluorescence intensity over nontreated control at the peak response time of 1 h after treatment. C, A549 cells were stably transfected with SOD, GPx, or control plasmid. Clonal lines expressing varying degrees of antioxidant enzymes were selected by immunoblotting using Abs specific for SOD and GPx. D, Transfectant lines were treated with FasL (100 ng/ml) for 12 h and analyzed for apoptosis. E, Transfectant lines were treated with FasL (100 ng/ml) and analyzed for DHE and DCF fluorescence intensities at 1 h posttreatment. Representative histograms of DHE and DCF measurements in the treated cells are shown. Plots are mean ± SD (n > 3), *, p < 0.05 vs nontreated control. #, p < 0.05 vs FasL-treated control. CAT indicates catalase.
Sigma-Aldrich), and rabbit polyclonal anti-S-nitrosocysteine Ab (N5411, 1:500, Sigma-Aldrich). After three washes with TBST, the membrane was incubated with peroxidase-conjugated isotype-specific secondary Abs (1: 500, Santa Cruz Biotechnology) for 1 h at room temperature and washed with 0.05% Tween 20 in PBS. Immunoreactive proteins were detected by chemiluminescence (SuperSignal West Pico, Pierce Biotechnology) and quantified by densitometry using UN-SCAN-IT digitizing software (Silk Scientific). Mean densitometry data from independent experiments were normalized to the control.

**Immunoprecipitation**

Cells were washed with cold PBS and lysed in lysis buffer at 4°C for 30 min. Cell lysates were centrifuged at 14,000 g for 15 min, and the supernatants were collected and analyzed for protein content. Samples containing 60 μg of protein were incubated with 12 μl of anti-FLIP agarose beads diluted with 12 μl of G-protein beads for 6 h at 4°C. The immune complexes were washed three times with 20 volumes of lysis buffer, resuspended in 2× Laemmli sample buffer, and heated at 95°C for 5 min. Immunoprecipitates containing 20 μg of protein were separated by 10% SDS-PAGE and analyzed by Western blotting as described.

**Statistical analysis**

The data represent means ± SD from three or more independent experiments. Statistical analysis was performed by Student's t test at a significance level of p < 0.05.

**Results**

**Induction of apoptosis and caspase activation by FasL in lung epithelial cells**

To study the regulatory mechanisms of Fas-induced apoptosis, we first characterized the apoptotic response to FasL treatment in primary AE2 cells from rats. The cells were treated with varying concentrations of FasL (0–100 ng/ml), and apoptosis was examined by nuclear DNA fragmentation and caspase activation assays. As shown in Fig. 1A, FasL treatment caused a dose-dependent increase in apoptotic cell death over nontreated control, as determined by Hoechst assay. The apoptotic cells exhibited condensed and/or fragmented nuclei with intense nuclear fluorescence (Fig. 1A). ELISA-based assay of apoptosis similarly showed a dose-dependent effect of FasL on DNA fragmentation (Fig. 1C). Caspase-8 activation, which is indicative of death receptor-mediated apoptosis (25, 26), was also induced by the FasL treatment, as evidenced by the cleavage of its inactive precursor form into active fragments (Fig. 1D). Addition of caspase-8 inhibitor (Z-IETD-FMK) potentely inhibited apoptosis and caspase-8 activation induced by FasL (Fig. 1, E and F), indicating the role of caspase-8 in epithelial cell death induced by FasL. Treatment of the cells with N-acetylcysteine (NAC), a general antioxidant, also inhibited FasL-induced cell death and caspase-8 activation (Fig. 1, E and F), suggesting the role of ROS in the apoptotic process.

**Role of ROS in FasL-induced apoptosis**

To determine the role of specific ROS in FasL-induced apoptosis of lung epithelial cells, AE2 cells were treated with FasL in the presence or absence of SOD (O2− scavenger), catalase (H2O2 scavenger), or sodium formate (OH− scavenger), and apoptosis was examined by Hoechst assay. Fig. 2A shows that all test scavengers were able to inhibit FasL-induced apoptosis in a dose-dependent manner, with catalase and SOD being the most effective. These results suggest that multiple ROS are involved in the apoptotic process and that H2O2 and O2− play a major role. To confirm the formation of ROS and their inhibition by the antioxidant enzymes in the treated cells, cellular ROS levels were determined using DHE and DCF-DA as fluorescent probes. Fig. 2B shows that FasL was able to induce superoxide and peroxide formation, as indicated by the increase in cellular DHE and DCF fluorescence intensities, respectively. Addition of SOD strongly inhibited FasL-induced DHE fluorescence, whereas catalase and sodium formate were ineffective, indicating the specificity of superoxide detection in the experiment. Analysis of peroxide formation by DCF fluorescence indicates inhibition of FasL-induced peroxide formation by catalase, but not by SOD or sodium formate. Interestingly, SOD increased DCF fluorescence intensity in FasL-treated cells, suggesting increased peroxide formation via dismutation of superoxide in the treated cells.

To confirm the role of superoxide and peroxide in FasL-induced cell death, we analyzed apoptotic and ROS responses to FasL treatment in SOD- and GPX-transfected cells. Because primary AE2 cells were refractory to gene transfection, we used alveolar epithelial A549 cells to aid gene transfer study. The cells were stably transfected with SOD, GPX, or control plasmid, and they were analyzed for antioxidant enzyme expression by Western blotting.
Two clonal lines from each of the SOD and GPx transfections were selected based on their relative antioxidant enzyme expression (Fig. 2C). The clonal lines were treated with FasL and analyzed for apoptosis. Fig. 2D shows that the SOD- and GPx-overexpressing lines exhibited significantly reduced apoptosis in response to FasL treatment as compared with the control line. The clonal lines that exhibited higher SOD and GPx expression also showed a more substantial reduction in apoptotic response to FasL treatment, consistent with our earlier data. Flow cytometric analysis of ROS generation also showed a substantial reduction in FasL-induced DHE and DCF fluorescence intensities in the SOD- and GPx-overexpressing lines as compared with the control line (Fig. 2E). Together, these results indicate the role of $O_2^-$ and $H_2O_2$ in FasL-induced apoptosis of lung epithelial cells.

NADPH oxidase is a key source of ROS generation induced by FasL.

To determine the source of ROS generation induced by FasL, cells were treated with FasL in the presence or absence of diphenylene iodonium (DPI), a known inhibitor of NADPH oxidase (27, 28), or rotenone, an inhibitor of mitochondrial electron transport chain (28, 29), and their effects on apoptosis and ROS generation were examined. The results show that DPI strongly inhibited FasL-induced apoptosis and ROS generation, whereas rotenone showed weak inhibitory effects (Fig. 3, A and B). Addition of Z-IETD-FMK, which was shown to inhibit FasL-induced caspase-8 activation and apoptosis (Fig. 1), had no significant effect on FasL-induced ROS generation (Fig. 3B). This result is consistent with a previous study showing that caspase inhibition was unable to inhibit ROS generation induced by death receptor activation (30). Together, our results indicate that NADPH oxidase is the primary source of ROS generation and that these ROS act upstream of the caspase-8 activation pathway.

To further determine the role of NADPH oxidase in FasL-induced ROS generation and apoptotic cell death, A549 cells were stably transfected with Rac1N17 or control plasmid, and their effects on FasL-induced ROS generation and apoptosis were determined. Rac1 is a small GTP-binding protein known to be involved in the activation of NADPH oxidase and generation of $O_2^-$ in various cell types (31–33). Fig. 3C shows that cells transfected with the Rac1N17 plasmid expressed a high level of the protein as compared with vector-transfected control cells. ROS generation, as analyzed by DHE fluorescence, was substantially reduced in the dominant-negative cells as compared with control cells after FasL treatment (Fig. 3D). These results indicate the role of Rac1-mediated ROS generation in FasL-induced apoptosis of lung epithelial cells.

Effect of FasL treatment on apoptosis-regulatory proteins

To determine the mechanism of apoptosis regulation by ROS in FasL-treated cells, we examined by immunoblotting the expression levels of key proteins known to be involved in Fas death signaling, including Fas, FADD, and FLIP. Among these, only the level of FLIP is down-regulated by FasL. Cells were treated with FasL (100 ng/ml) and analyzed for FLIP by Western blotting. Values are means ± SD ($n > 3$). * $p < 0.05$ vs nontreated control.

FIGURE 4. Effect of FasL treatment on apoptosis-regulatory proteins. A, AE2 cells were treated with FasL (0–100 ng/ml) for 12 h. The cells were then washed with ice-cold PBS and extracted with lysis buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with Abs specific for Fas, FADD, and FLIP. Blots were reprobed with β-actin Ab to confirm equal loading of the samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiments (one of which is shown here) were normalized to the results obtained in cells in the absence of FasL (control). B, Time-dependent down-regulation of FLIP by FasL. Cells were treated with FasL (100 ng/ml) for various times and analyzed for FLIP by Western blotting. Values are means ± SD ($n > 3$). * $p < 0.05$ vs nontreated control.

FIGURE 5. Effects of FLIP siRNA knockdown and gene overexpression on FasL-induced apoptosis and ROS generation. A, AE2 cells were transfected with siFLIP or siCONTROL oligonucleotides as described in Materials and Methods. The cells were characterized for FLIP protein expression by Western blotting. β-actin was used as a loading control. B, Transfected cells were treated with varying doses of FasL (0–100 ng/ml) for 12 h and analyzed for apoptosis by Hoechst assay. C, A549 cells were stably transfected with FLIP or control plasmid. The cells were analyzed for FLIP protein levels by immunoblotting. D, Transfected cells were treated with varying doses of FasL (0–100 ng/ml) for 12 h and analyzed for apoptosis. E, siFLIP, FLIP, and control transfectants were treated with FasL (100 ng/ml) and analyzed for DHE and DCF fluorescence after 1 h. Plots are means ± SD ($n = 4$). * $p < 0.05$ vs FasL-treated controls.
FLIP was affected by the FasL treatment in a dose- and time-dependent manner (Fig. 4). Only FLIPL could be detected in this study, whereas the short isoform (FLIPS) was undetectable.

To determine the role of FLIP in FasL-induced apoptosis of lung epithelial cells, AE2 and A549 cells were transfected with siFLIP and full-length FLIP plasmid, respectively, and their effects on FLIP protein expression and cell death response to FasL were examined. Fig. 5, A and B shows that siFLIP-transfected cells exhibited reduced expression of FLIP and were more susceptible to FasL-induced apoptosis than were siCONTROL-transfected cells. Transfection of cells with FLIP plasmid increased FLIP protein expression and reduced cell death response to FasL treatment as compared with vector-transfected control (Fig. 5, C and D). Transfection of the cells with FLIP and siFLIP also resulted in a corresponding decrease and increase in cellular ROS levels following FasL treatment as compared with their respective controls (Fig. 5E). Taken together, these results indicate a negative regulatory role of FLIP in FasL-induced apoptosis and ROS generation in lung epithelial cells.

Regulation of FLIP by ROS

FLIP has been shown to be regulated by the ubiquitin-proteasome pathway under different conditions (34–36). To determine whether this pathway is involved in the down-regulation of FLIP by FasL in the test cell system, cells were treated with lactacystin, a highly specific proteasome inhibitor, and its effect on FasL-induced FLIP down-regulation was determined by Western blotting. Fig. 6A shows that lactacystin completely inhibited FLIP down-regulation, indicating the role of proteasomal degradation in FasL-induced down-regulation of FLIP in lung epithelial cells.

To determine the role of ROS in FLIP down-regulation, cells were treated with FasL in the presence or absence of SOD and catalase, and their effect on FLIP protein expression was determined. Fig. 6A shows that treatment of the cells with catalase completely inhibited FasL-induced FLIP down-regulation, whereas SOD treatment showed a partial inhibitory effect. These results were confirmed in GPx- and SOD-overexpressing lines, which exhibited a relatively constant level of Fas expression in the presence or absence of FasL activation (Fig. 6B). The results indicate that H$_2$O$_2$ is a major oxidative species responsible for FLIP down-regulation by FasL. Ubiquitination studies further showed that catalase strongly inhibited FasL-induced ubiquitination of FLIP (Fig. 6C), supporting the role of H$_2$O$_2$ in ubiquitin-mediated down-regulation of FLIP through the proteasome pathway. Direct treatment of the cells with H$_2$O$_2$ similarly induced FLIP ubiquitination, the effect that was inhibited by catalase (Fig. 6D), further supporting the role of this oxidative species in FLIP down-regulation.

Regulation of FLIP by superoxide

The observation that SOD inhibited FLIP down-regulation is somewhat surprising because SOD treatment had no inhibitory effect on peroxide formation, but instead it increased the peroxide level in FasL-treated cells (Fig. 2C). Because previous
studies have shown that FLIP ubiquitination is negatively regulated by NO, which is induced by FasL (37), and because NO is known to interact with O2•− (38, 39), we investigated this potential interaction as a possible mechanism of FLIP regulation by O2•−. Cells were treated with FasL (100 ng/ml) for 3 h and analyzed for NO levels by flow cytometry using DAF-DA as a probe. B, Cells were similarly treated with the test agents and analyzed for FLIP protein levels by Western blotting after 12 h. C, Cells were similarly treated and analyzed for FLIP S-nitrosylation by immunoprecipitation at 3 h posttreatment. Cell lysates were immunoprecipitated with anti-FLIP Ab, and the immune complexes were analyzed by Western blot using S-nitrosocysteine Ab. The density of nitrosylated FLIP bands was normalized against non-treated control band. Plots are means ± SD (n = 3). *p < 0.05 vs nontreated control. **p < 0.05 vs FasL-treated control. #p < 0.05 vs FasL/SOD-treated control.

**FIGURE 7.** Effects of SOD treatment on NO production and FLIP nitrosoylation. A, AE2 cells were either left untreated or pretreated for 1 h with SOD (1000 U/ml) in the presence or absence of NO inhibitor PTIO (100 μM). The cells were then treated with FasL (100 ng/ml) for 3 h and analyzed for NO levels by flow cytometry using DAF-DA as a probe. B, Cells were similarly treated with the test agents and analyzed for FLIP protein levels by Western blotting after 12 h. C, Cells were similarly treated and analyzed for FLIP S-nitrosylation by immunoprecipitation at 3 h posttreatment. Cell lysates were immunoprecipitated with anti-FLIP Ab, and the immune complexes were analyzed by Western blot using S-nitrosocysteine Ab. The density of nitrosylated FLIP bands was normalized against nontreated control band. Plots are means ± SD (n = 3). *p < 0.05 vs nontreated control. **p < 0.05 vs FasL-treated control. #p < 0.05 vs FasL/SOD-treated control.

ROS AND Fas DEATH SIGNALING

ROS have long been known to be involved in the regulation of apoptosis induced by various pathologic and physiologic stimuli. However, their role in Fas-induced apoptosis and its regulatory mechanisms are unclear. In the present study, we showed that FasL-induced apoptosis of lung epithelial cells is associated with the generation of multiple ROS and that inhibition of these ROS by antioxidant enzymes catalase and SOD effectively inhibited the apoptotic effect of FasL (Fig. 2A). Overexpression of GPx and SOD by gene transfection similarly inhibited FasL-induced apoptosis (Fig. 2D), supporting the role of H2O2 and O2•− in the death signaling process. These results are consistent with previous reports showing the proapoptotic role of H2O2 and O2•− in various cell types (40–42). In Jurkat T cells, Gulbins et al. (20) and Sato et al. (21) suggested O2•− as a mediator of Fas death signaling, whereas Medan et al. (22) showed H2O2 and OH• as key mediators in macrophages. In contrast, Hug et al. (23) found no requirement of ROS in fibroblasts overexpressing Fas, while Atonis et al. (43) showed an antiapoptotic role of ROS in Fas-activated Jurkat cells. These studies suggest that the apoptotic response to Fas activation of ROS is cell type specific and may be determined by the expression levels of antioxidant enzymes. O2•− could be the early ROS produced by cells in response to Fas activation, and depending on the expression level of its scavenging enzyme, SOD determines the degree of sensitivity to Fas-induced apoptosis. The expression of SOD has been shown to be up-regulated following death receptor activation through an NF-κB-dependent pathway (44, 45). In NF-κB-deficient cells, ROS generation is enhanced upon treatment with the death ligand TNF-α, and ectopic expression of SOD inhibits this effect (44, 45), suggesting the role of NF-κB in SOD-mediated inhibition of ROS. The induction of ROS by TNF-α has also been shown to be dependent on JNK activation (46), which is negatively regulated by FLIP (47). Because FLIP is known to activate NF-κB (48, 49), its increased expression could lead to SOD up-regulation and decreased O2•− formation. Thus, in addition to being affected by ROS, FLIP also regulates ROS through JNK and NF-κB activation, forming a feedback loop that controls the death signaling. Because ROS generation induced by FasL was shown to be mediated through NADPH oxidase, it is also possible that FLIP may have a direct effect on this enzyme and thereby regulate the ROS and apoptotic responses to Fas activation. It is likely that the difference in the expression levels of SOD and other ROS regulatory proteins described herein determine the variable ROS responses to Fas activation observed in the previous studies. Should the level of SOD be high, most of the O2•− radicals generated would be rapidly converted to H2O2, thereby putting pressure on the catalase and peroxidase systems. It is also conceivable that potential suboptimal expression levels of catalase and peroxidase could have resulted in increased H2O2 levels. Such elevated levels, detected in our experiments, could in turn initiate a cascade of cellular signaling events, including caspase activation and apoptosis.
The results of this study also show that NADPH oxidase is a key source of ROS generation induced by FasL. Supporting this notion is the evidence that DPI, a known inhibitor of NADPH oxidase (27, 28), strongly inhibited ROS generation and apoptosis induction by FasL, whereas rotenone, a known inhibitor of mitochondrial ROS (28, 29), had minimal effects (Fig. 3). The mechanism by which FasL activates NADPH oxidase is unclear but was shown to involve Rac1 activation because its inhibition by Rac1N17 expression inhibited the induction of O$_2^-$ by FasL (Fig. 3D). Rac1 protein is known to be involved in the assembly of the NADPH oxidase system, which is responsible for transferring electrons from NADPH to molecular oxygen with the subsequent production of O$_2^-$. The activation of Rac1 by Fas has also been shown to be mediated through Ras signaling (20), which is activated upon Fas receptor ligation (51). We have further shown that inhibition of Rac1 by dominant-negative expression inhibited apoptotic cell death induced by FasL (Fig. 3D). As indicated earlier, O$_2^-$ can be dismutated by SOD to form H$_2$O$_2$, which could serve as a key mediator of FasL-induced apoptosis. This was demonstrated by the ability of the peroxide-scavenging enzymes catalase and GPx to inhibit FasL-induced apoptosis (Fig. 2).

The results of this study also show that ROS mediate the apoptotic effect of FasL, at least in part through down-regulation of FLIP. FasL induced down-regulation of FLIP without having a significant effect on FADD and Fas protein expression (Fig. 4). FLIP is known to render cells resistant to death receptor-mediated apoptosis in various cell types (13, 14, 52–54), and its elevated expression has been associated with tumor cells that escape from immune surveillance (55). Consistent with these earlier reports, our gene overexpression and siRNA knockdown studies indicate the inhibitory role of FLIP in FasL-induced apoptosis of lung epithelial cells (Fig. 5). Down-regulation of FLIP by FasL was shown to be dependent on ROS because this effect was inhibited by the antioxidant enzymes catalase, GPx, and SOD (Fig. 6).

The mechanism by which ROS mediates the down-regulation of FLIP by FasL was shown to involve ubiquitin-proteasomal degradation because inhibition of ROS by antioxidants inhibited the ubiquitination and proteasomal degradation of FLIP by FasL (Fig. 6). Direct treatment of the cells with H$_2$O$_2$ similarly induced ubiquitination and proteasomal degradation of FLIP by FasL (Fig. 5). Down-regulation of FLIP by FasL was shown to be dependent on ROS because this effect was inhibited by the antioxidant enzymes catalase, GPx, and SOD (Fig. 6).

In addition to ROS, FLIP has also been shown to be under the regulation of NO. However, unlike ROS, NO plays a protective role in FLIP down-regulation through its ability to stabilize the protein via S-nitrosylation, which prevents its degradation through the ubiquitin-proteasomal pathway (36). Our results show that this process is linked to the ROS regulatory mechanism because inhibition of ROS by the antioxidant SOD increased the cellular levels of NO in FasL-treated cells (Fig. 7A). SOD treatment also increased NO-mediated S-nitrosylation of FLIP, which was reversed by cotreatment with the NO inhibitor PTIO (Fig. 7C). PTIO also inhibited the SOD effect on FLIP protein expression in FasL-treated cells (Fig. 7B). Taken together, these results indicate the interactive role of NO and O$_2^-$ in the regulation of FLIP, thus revealing a novel pathway of apoptosis regulation in Fas-induced cell death.

In conclusion, we have shown that ROS play an important role in the regulation of apoptosis induced by FasL in lung epithelial cells. The mechanism by which ROS regulate apoptosis involves down-regulation of FLIP, which functions as a suppressor of Fas death signaling in these cells. H$_2$O$_2$ is a major oxidative species responsible for FLIP down-regulation via ubiquitin-proteasomal degradation, while O$_2^-$ serves as a source of H$_2$O$_2$ and as a negative regulator of NO. Because aberrant expression of ROS and NO has been associated with several human disorders, the results of this study could have important implications in the understanding of disease pathogenesis and treatment of associated apoptosis disorders.

Disclosures
The authors have no financial conflicts of interest.

References


