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Nitric Oxide Negatively Regulates Fas CD95-induced Apoptosis through Inhibition of Ubiquitin-Proteasome-mediated Degradation of FLICE Inhibitory Protein*

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Stimulation of cell surface Fas (CD95) results in recruitment of cytoplasmic proteins and activation of caspase-8, which in turn activates downstream effector caspases leading to programmed cell death. Nitric oxide (NO) plays a key role in the regulation of apoptosis, but its role in Fas-induced cell death and the underlying mechanism are largely unknown. Here we show that stimulation of the Fas receptor by its ligand (FasL) results in rapid generation of NO and concomitant decrease in cellular FLICE inhibitory protein (FLIP) expression without significant effect on Fas and Fas-associated death domain (FADD) adapter protein levels. FLIP down-regulation as well as caspase-8 activation and apoptosis induced by FasL were all inhibited by the NO-liberating agent sodium nitroprusside and dipropylenetriamine NONOate, whereas the NO synthase inhibitor aminoguanidine and NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO) had opposite effects, indicating an anti-apoptotic role of NO in the Fas signaling process. FasL-induced down-regulation of FLIP is mediated by a ubiquitin-proteasome pathway that is negatively regulated by NO. *S*-nitrosylation of FLIP is an important mechanism rendering FLIP resistant to ubiquitination and proteasomal degradation by FasL. Deletion analysis shows that the caspase-like domain of FLIP is a key target for *S*-nitrosylation by NO, and mutations of its cysteine 254 and cysteine 259 residues completely inhibit *S*-nitrosylation, leading to increased ubiquitination and proteasomal degradation of FLIP. These findings indicate a novel pathway for NO regulation of FLIP that provides a key mechanism for apoptosis regulation and a potential new target for intervention in death receptor-associated diseases.

Apoptosis is a form of programmed cell death that serves to eliminate unwanted cells and is essential for the maintenance of tissue homeostasis. It can be triggered by extracellular signals via death receptors that belong to the tumor necrosis factor receptor family (1, 2). In particular, Fas (CD95/Apo-1) plays a crucial role in maintaining the immune system by inducing apoptosis of immune cells as well as in killing harmful cells such as cancerous cells (3). Defects in the apoptosis regulatory mechanisms of the Fas/FasL system often result in lymphoproliferative disorders and autoimmune diseases (4–6) as well as tumor outgrowth *in vivo* (7). The Fas/FasL system also plays important roles in various apoptosis conditions such as those evoked by anti-tumor agents, viral infections, and irradiation (8–11).

Activation of the Fas receptor by FasL triggers a complex cascade of intracellular events that requires Fas-associated death domain (FADD)[3] adapter protein and the formation of death-inducing signaling complex, leading to caspase-8 activation and apoptosis (12, 13). Although FasL binding to its receptor is required for such activation, Fas surface expression does not necessarily render cells susceptible to FasL-induced cell death, indicating that inhibitors of the apoptosis signaling pathway exist and play a role (3, 4). FLICE-inhibitory protein (FLIP) is a key apoptosis regulatory protein of the death receptor-mediated pathway. FLIP inhibits apoptotic signaling by interfering with the binding of caspase-8 to FADD at the death-inducing signaling complex (14, 15). FLIP is involved in rendering cells resistant to death receptor-mediated apoptosis in various cell types (14–19), and elevated expression of FLIP is associated with tumor cells that can escape from immune surveillance *in vivo* (7). Furthermore, down-regulation of FLIP by cytotoxic agents has been shown to sensitize cells to Fas-mediated apoptosis (20).

Endogenously produced nitric oxide (NO) synthesized from L-arginine by NO synthase is a mediator of a variety of physiological and pathological processes (21, 22). NO has been shown to possess both pro- and anti-apoptotic functions depending on cell type, redox status, and type and dose of NO-modulating agents (23, 24). The apoptosis-inducing effect of NO was attributed to its ability to induce oxidative stress and caspase activation (25). On the other hand, endogenous NO production or exposure to appropriate amounts of NO has been reported to inhibit apoptosis in several cell types (26, 27). Likewise, NO has been shown to inhibit cell death induced by a variety of agents including chemotherapeutic agents, viral infections, and death ligands (24, 28, 29). The mechanisms by which NO regulates apoptosis are not well understood, but both mitochondrial and death receptor pathways of apoptosis are known to be involved (30, 31). In the present study we attempted to investigate the role of NO in Fas receptor-mediated apoptosis and determine its regulatory mechanisms using pharmacological and genetic manipulation approaches. Our findings demonstrate an important role of NO in FLIP regulation and its anti-apoptotic function in Fas death signaling. The mechanism by which NO regulates FLIP involves

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[3] The abbreviations used are: FADD, Fas-associated death domain; FLIP, FLICE-inhibitory protein; TRAF-2, tumor necrosis factor receptor-associated factor-2; AG, aminoguanidine; SNP, nitroprusside; PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; CHAPS, 3-[(3-cholamidiopropyl)-1] propane sulfonate; DPTA, dipropylenetriamine NONOate.

S-nitrosylation and its inhibition of ubiquitin-proteasomal degradation, thus revealing the existence of a novel mechanism of cell death regulation that might be exploited in death receptor-induced apoptosis of neoplastic and immune cells.

MATERIALS AND METHODS

Cells and Reagents—The human bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (Mannassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 5% fetal bovine serum, 2 mM L-glutamine, 20 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 environment at 37 °C. Recombinant FasL (SuperFasL), monoclonal antibody against FLIP (Dave-2), and the NO donor DPTA NONOate were purchased from Alexis Biochem (San Diego, CA). Antibodies for Fas, FADD, and peroxidase-labeled secondary antibodies to IgG and protein A-agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for ubiquitin, S-nitrosocysteine, and β-actin were from Sigma. The NO donor sodium nitroprusside (SNP), NO inhibitors amino-guanidine (AG), 2-(4-carboxy-phenyl)-4,4,5,5 tetramethylimidazoline-1-oxy-3-oxide (PTIO), and caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone were from Sigma. The transfecting agent Lipofectamine Plus was purchased from Invitrogen, and the caspase-8 fluorometric substrate IETD-amino-4-methyl coumarin (AMC) was from Biovision Inc. (Mountain View, CA).

Plasmid Construction and Transfection—The pcDNA3-$FLIP_L$ plasmid was generously provided by Dr. C. Stehlik (West Virginia University Cancer Center, Morgantown, WV). The open reading frame of $FLIP_L$ and ubiquitin were amplified by high fidelity PCR (Stratagene) from the corresponding expressed sequence tags (ESTs) and cloned into pcDNA3 expression vectors containing the N-terminal Myc epitope tag. Myc-tagged $FLIP_{Cys/Ala}$ was generated using the QuikChange mutagenesis kit (Stratagene). Authenticity of all constructs was verified by DNA sequencing. Transient transfection was performed using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions at 80–90% confluency. The amount of DNA was normalized in all transfection experiments with pcDNA3. Expression of proteins was verified by Western blotting or immunoprecipitation.

Caspase and Apoptosis Assays—Caspase-8 activity was determined by fluorometric assay using the substrate IETD-AMC, which is specifically cleaved by the enzyme at the Asp residue to release the fluorescent leaving group, AMC. Cell extracts containing 50 μg of protein were incubated with 100 mM HEPES, pH 7.4, containing 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, and 50 μM caspase substrate in a total reaction volume of 0.25 ml. The reaction mixture was incubated for 60 min at 37 °C. At the end of incubation, the liberated fluorescent group AMC was determined fluorometrically at the excitation and emission wavelengths of 380 and 460 nm, respectively. Apoptosis was determined by incubating the cells with 10 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) for 30 min and scoring the percentage of cells having intensely condensed chromatin and/or fragmented nuclei by UV microscopy using the Pixera software (Leica, Germany).

NO Detection—The production of NO was assessed by flow cytometry using a fluorescent probe, 4,5-diaminofluorescein diacetate (Molecular Probes) according to the manufacturer's instruction. Briefly, cells (1×10^6/ml) were loaded with 10 μM 4,5-diaminofluorescein diacetate for 30 min at 37 °C, after which they were thoroughly washed. A FACSort (BD Biosciences) flow cytometer equipped with a 488-nm argon ion laser and supplied with the Cell Quest software was applied to measure NO levels in the cells. Signals were obtained using a 538-nm bandpass filter. Each determination is based on mean fluorescence intensity of 5000 cells.

Western Blot Analysis—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 NaCl, 10% glycerol, 1 mM Na_3VO_4, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Roche Applied Science) for 20 min on ice. After insoluble debris was pelletted by centrifugation at 14,000 × *g* for 15 min at 4 °C, the supernatants were collected and determined for protein content using the Bradford method (Bio-Rad). Proteins (40 μg) were resolved on a reducing 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in Tris-buffered Tween (25 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.05% Tween 20) and incubated with the appropriate primary antibodies at 4 °C overnight. Membranes were washed 3 times with Tris-buffered Tween for 10 min and incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) and quantified using analyst/PC densitometry software (Bio-Rad). Mean densitometry data from independent experiments were normalized to result in cells in the control. The data were presented as the mean ± S.D. and analyzed by Student's *t* test.

Immunoprecipitation—Cells were washed after treatments with ice-cold phosphate-buffered saline and lysed in lysis buffer at 4 °C for 30 min. After centrifugation at 14,000 × *g* for 15 min at 4 °C, the supernatants were collected and determined for protein content. Cell lysates containing 60 μg of protein were incubated with 12 μl of anti-Myc-agarose beads (Santa Cruz Biotechnology) diluted with 12 μl of Sepharose for 6 h at 4 °C. The immune complexes were then washed 3 times with 20 volumes of lysis buffer, resuspended in 2× Laemmli sample buffer, and boiled at 95 °C for 5 min. Immunoprecipitates containing ~20 μg of protein equivalents were separated by 10% SDS-PAGE and analyzed by Western blot as described.

RESULTS

FasL Induces Apoptosis and Caspase-8 Activation in BEAS-2B Cells—FasL has been reported to induce apoptosis in sensitive cells, including lung epithelial cells (32–36). To study the role of NO in Fas-mediated apoptosis, we first characterized cell death response to FasL treatment in lung epithelial BEAS cells using Hoechst 33342 and caspase assays. Treatment of the cells with FasL caused a dose-dependent increase in cell apoptosis over control levels, as indicated by increased nuclear fluorescence and chromatin condensation and fragmentation (Fig. 1). Approximately 8% of the treated cells showed apoptotic nuclear morphology at the FasL concentration of 50 ng/ml with the cell death response exceeding 25% at 250 ng/ml. Significant apoptotic response was observed as early as 6 h and peaked at about 16 h post-treatment (data not shown). Because FasL is known to induce apoptosis through a caspase-8-dependent pathway (3, 4), we investigated the effect of FasL treatment on caspase-8 activity using fluorogenic caspase-8 substrate IETD-AMC. Consistent with the Hoechst apoptosis assay, our results indicated that FasL was able to increase the activity of caspase-8 in BEAS cells in a dose-dependent manner (Fig. 1*B*). The caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (10 μM) potently suppressed FasL-induced apoptosis and caspase-8 activation (data not shown), further supporting an apoptotic mechanism.

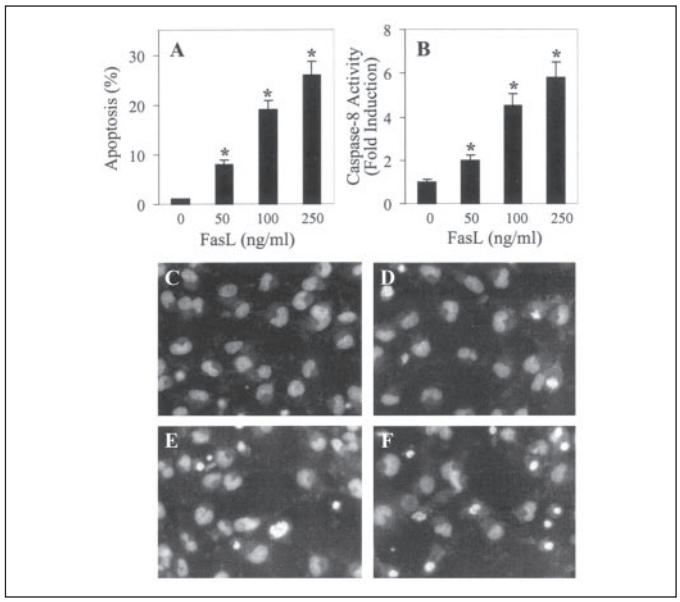

FIGURE 1. **FasL induces apoptosis and caspase-8 activation in human lung epithelial BEAS cells.** *A*, subconfluent (90%) monolayers of BEAS cells were exposed to FasL (0–250 ng/ml) for 16 h and analyzed for apoptosis by Hoechst 33342 assay. *B*, fluorometric assay of caspase-8 activity in cells treated with FasL (0–250 ng/ml) for 3 h. Cell lysates (50 μg of protein) were prepared and determined for caspase-8 activity using the fluorometric substrate IETD-AMC. Data are the mean ± S.D. ($n = 4$). *, $p < 0.05$ *versus* non-treated control. *C–F*, morphologic analysis of apoptosis by Hoechst assay. Cells were treated with 0, 50, 100, and 250 ng/ml of FasL for 16 h. Apoptotic cells exhibit shrunken nuclei with bright nuclear fluorescence. Original magnification, ×400.

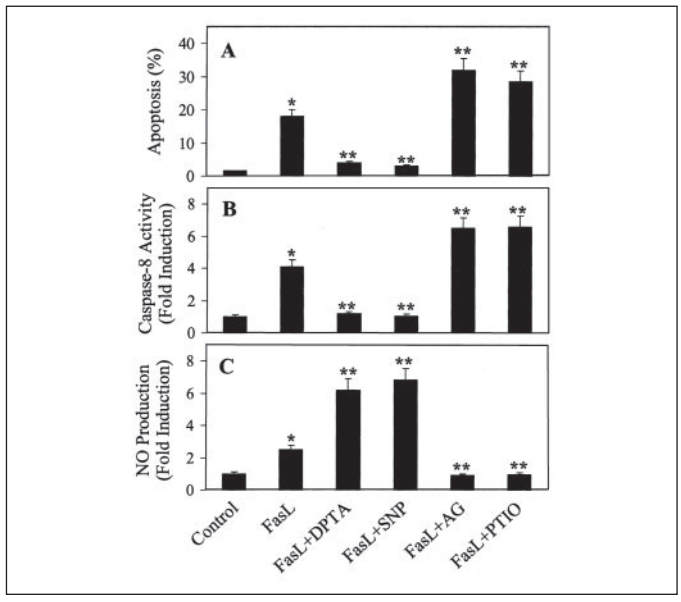

FIGURE 2. **Effects of NO modulators on FasL-induced apoptosis, caspase-8 activation, and NO production.** *A*, subconfluent (90%) monolayers of BEAS cells were pretreated with the NO donor SNP (300 μg/ml) or DPTA NONOate (200 μM) or with the NO inhibitor AG (100 μg/ml) or PTIO (100 μM) for 1 h. The cells were then treated with FasL (100 ng/ml) for 16 h and analyzed for apoptosis by Hoechst assay. *B*, fluorometric analysis of caspase-8 activity, determined at 3 h after FasL treatment. *C*, flow cytometric analysis of NO production by DAF fluorescence at 1 h after FasL treatment. Cells were treated with FasL and NO modulators as described. Values are relative DAF fluorescence increase over control level. Plots are the mean ± S.D. ($n \geq 3$). *, $p < 0.05$ *versus* non-treated control; **, $p < 0.05$ *versus* FasL-treated control.

Nitric Oxide Inhibits FasL-induced Apoptosis and Caspase-8 Activation—NO has been reported to have both pro- and anti-apoptotic effects on cells depending on cell type, redox status, and stimulating agents (23–25). In this study we found that NO has an inhibitory effect on FasL-induced cell death in lung epithelial BEAS cells. Co-treatment of the cells with FasL and NO donor, SNP or DPTA NONOate, significantly inhibited apoptosis compared with the FasL-treated control (Fig. 2*A*). In contrast, the NO inhibitor AG or PTIO promoted this effect (Fig. 2*A*). The NO modulators, when used alone at the indicated concentrations, had no significant effect on cell death (not shown), indicating that FasL was required for the induction of apoptosis under the test conditions. To test whether the apoptosis-modulating effect of NO was mediated through the death receptor signaling pathway, cells were similarly treated with FasL and NO modulators, and cell lysates were prepared and analyzed for caspase-8 activity. Fig. 2*B* shows that the NO donors, SNP and DPTA NONOate, were able to inhibit caspase-8 activation by FasL, whereas the NO inhibitors, AG and PTIO, promoted the FasL effect.

Because AG is an inhibitor of inducible NO synthase, our results suggest that FasL was able to induce NO production, and AG was able to inhibit it. To test this possibility and to determine the NO modulating effect of other test agents, BEAS cells were treated with FasL in the presence or absence of various NO donors and inhibitors, and their effect on cellular NO levels was determined by flow cytometry using NO-specific probe 4,5-diaminofluorescein diacetate. As expected, FasL was able to increase DAF fluorescence intensity over control levels, and the NO inhibitors AG and PTIO suppressed this signal (Fig. 2*C*). In contrast, the NO donors SNP and DPTA NONOate promoted the NO-inducing effect of FasL.

FasL Induces FLIP Down-regulation and Its Inhibition by Nitric Oxide—In searching for a mechanism that might explain the apoptosis regulatory effect of NO on FasL-induced cell death, we examined by immunoblotting the expression levels of key proteins known to be relevant to the mechanisms of Fas signaling, including the Fas death receptor, the adapter protein FADD, and the anti-apoptotic FLIP, which is known to bind caspase 8 and FADD and suppress apoptosis induction by death ligands (14, 15). Among these, only the level of FLIP was affected by the FasL treatment in a dose- and time-dependent manner (Figs. 3, *A* and *B*). In the cell line tested, only the long isoform of FLIP ($FLIP_L$) could be detected, whereas the short $FLIP_S$ protein was undetectable. Because FLIPs have been reported to be down-regulated via the ubiquitin proteasome-dependent pathway under different conditions, including peroxisome proliferator-activated receptor ligand and p53 activation, chemotherapeutic administration, and adenoviral infection (37–40), we therefore investigated whether the down-regulation of FLIP by FasL is also mediated by this pathway. Cells were treated with lactacystin, a highly specific proteasome inhibitor, and its effect on FasL-induced FLIP down-regulation was examined by Western blot analysis. Fig. 3*A* shows that lactacystin was able to inhibit FLIP down-regulation, indicating proteasomal degradation of FLIP by FasL. The result was confirmed by the fact that another proteasome inhibitor, MG132, also inhibited the decrease in FLIP expression caused by FasL (data not shown). These results along with subsequent data showing the effect of FasL on FLIP ubiquitination support the role of the ubiquitin-proteasome pathway in FasL-induced degradation of FLIP.

To test whether NO might modulate Fas signaling through FLIP, cells were treated with FasL in the presence or absence of NO donors or inhibitors, and their effect on FLIP expression levels was determined by immunoblotting. Fig. 3*B* shows that the addition of the NO donor SNP or DPTA NONOate strongly increased FLIP levels over FasL-treated control, whereas the NO inhibitor AG showed an opposite effect. These results along with our earlier finding on the protective effect of NO on FasL-induced cell death (Fig. 2) suggest that NO may mediate its anti-apoptotic effect by interfering with the FLIP degradation mechanism.

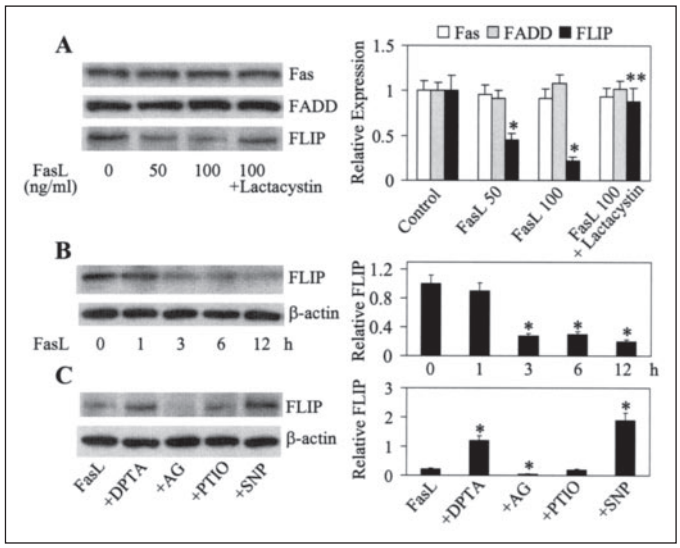

FIGURE 3. **Down-regulation of FLIP by FasL is modulated by NO.** *A*, Western blot analysis of FLIP in response to FasL treatment. Subconfluent monolayers of BEAS cells were treated with FasL (0–100 ng/ml) in the presence or absence lactacystin (10 μM) for 12 h. The cells were then washed with ice-cold phosphate-buffered saline and extracted with SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with antibodies specific for Fas, FADD, and FLIP. Blots were reprobed with β-actin antibody to confirm equal loading of samples. The 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 cells in the absence of FasL (control). Plots are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* control; **, $p < 0.05$ *versus* FasL-treated control. *B*, time-dependent effect of FasL treatment on FLIP expression. Cells were treated for the indicated time with FasL (100 ng/ml) and analyzed for FLIP by Western blot. *, $p < 0.05$ *versus* non-treated control ($n = 3$). *C*, effect of NO modulators on FasL-induced FLIP down-regulation. Cells were pretreated with the NO donor SNP (300 μg/ml) or DPTA NONOate (200 μM), or with the NO inhibitor AG (100 μg/ml) or PTIO (100 μM), for 1 h, after which they were treated with FasL (100 ng/ml) for 12 h and analyzed for FLIP by Western blot. *, $p < 0.05$ *versus* FasL-treated control ($n = 3$).

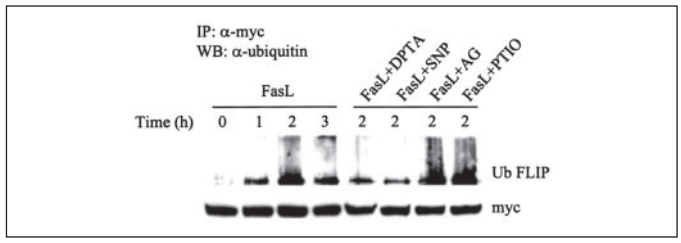

FIGURE 4. **NO regulation of FLIP is mediated through the ubiquitin pathway.** *A*, BEAS cells were transiently transfected with myc-FLIP and ubiquitin plasmids and then treated 1 day later with FasL (100 ng/ml) for the indicated times in the presence or absence of SNP (300 μg/ml), DPTA NONOate (200 μM), AG (100 μg/ml), or PTIO (100 μM). Lysates were immunoprecipitated (*IP*) by incubation with 12 μl of anti-Myc-agarose beads diluted with 12 μl of Sepharose for 6 h at 4 °C. The beads were washed, boiled, and subjected to 10% polyacrylamide gel electrophoresis. The separated proteins were analyzed by Western blot (*WB*) with antibody against ubiquitin (*Ub*). Blots were also probed with Myc antibody to confirm equal loading of samples.

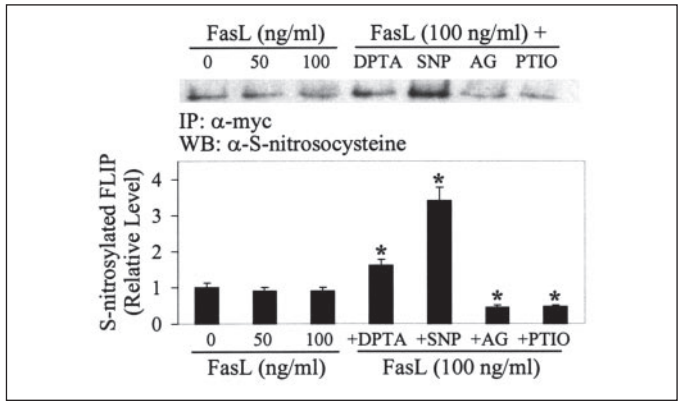

FIGURE 5. ***S*-Nitrosylation of FLIP by NO.** BEAS cells were transiently transfected with Myc-FLIP plasmid and treated 1 day later with FasL (0–100 ng/ml) in the presence or absence of SNP (300 μg/ml), DPTA NONOate (200 μM), AG (100 μg/ml), or PTIO (100 μM) for 2 h. Lysates were immunoprecipitated (*IP*) using Myc antibody and analyzed by Western blot (*WB*) using *S*-nitrosocysteine antibody. The density of *S*-nitrosylated FLIP bands was determined by densitometry and normalized against non-treated control band. Plots are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* FasL-treated control.

FasL Induces FLIP Ubiquitination and Its Inhibition by Nitric Oxide—Because the proteasome acts on proteins destined to be degraded by ubiquitination, we investigated whether FLIP ubiquitination is induced by FasL. Immunoprecipitation studies were performed in cells transiently transfected with plasmids encoding ubiquitin and Myc-tagged FLIP, and the resulting immune complexes were analyzed by SDS-PAGE immunoblotting using anti-ubiquitin antibody. Fig. 4 shows that in the absence of FasL stimulation, minimum ubiquitinated FLIP was produced. Upon FasL treatment, the ubiquitin-FLIP conjugate was greatly increased in a time-dependent manner (Fig. 4). This effect could be observed as early as 1 h and peaked at 2 h after FasL treatment. Pretreatment of the cells with NO donors, SNP, or DPTA NONOate potently inhibited FasL-induced ubiquitination of FLIP, whereas the NO inhibitors AG and PTIO enhanced this ubiquitination process (Fig. 4). These results suggest that NO was able to inhibit FLIP ubiquitination, thus preventing its degradation by the proteasome.

Nitric Oxide Induces S-Nitrosylation of FLIP and Inhibits Its Ubiquitination—Increasing evidence indicates that NO plays an important role in apoptosis through *S*-nitrosylation of several key apoptosis regulatory proteins (41, 42). To determine whether NO could nitrosylate FLIP, which has not previously been demonstrated, we performed immunoprecipitation experiments evaluating the effect NO on *S*-nitrosylation of FLIP. Cells expressing ectopic myc-FLIP were treated with FasL and NO modulators, and cell lysates were immunoprecipitated and analyzed by Western blot using anti-*S*-nitrosocysteine antibody. Fig. 5 shows that in the absence of NO modulators, FasL had minimal effect on *S*-nitrosylation of FLIP. However, upon the addition of the NO donors, SNP or DPTA NONOate, the nitrosylated level of FLIP was strongly increased. In contrast, the NO inhibitors, AG or PTIO, inhibited this nitrosylation. These results suggest that *S*-nitrosylation may be a key mechanism utilized by NO to regulate FLIP ubiquitination and proteasomal degradation.

The Caspase-like Domain Is a Target for S-Nitrosylation of FLIP—To study the mechanism of *S*-nitrosylation of FLIP, we first determined which domain(s) of FLIP is responsible for its nitrosylation. We constructed a series of FLIP deletion and mutation plasmids (Δ1-Δ4) and tested their effect on *S*-nitrosylation by NO (Fig. 6). Partial deletion of the caspase-like domain of FLIP (amino acids 329–480, Δ1) had no effect on *S*-nitrosylation induced by the NO donor SNP, whereas complete deletion of this domain (Δ2) as well as the death effector domain 2 (Δ3) strongly inhibited this effect when the plasmids were transfected into cells and subsequently treated with FasL (Fig. 6*B*). Immunoprecipitation and ubiquitination studies also showed that the NO donor SNP was able to inhibit FasL-induced ubiquitination of FLIP and its Δ1 mutant but not the Δ2 and Δ3 mutants (Fig. 7), indicating the protective effect of *S*-nitrosylation on FLIP ubiquitination. The results of this study also suggest that the amino acid sequence difference between Δ1 and Δ2 (or the amino acid sequence 233–328 of the caspase-like domain) is essential for *S*-nitrosylation of FLIP.

Cysteines 254 and 259 Are the Principal Target Sites for S-Nitrosylation of FLIP—To determine the target site(s) for *S*-nitrosylation of FLIP, we examined the amino acid sequence in the target region of caspase-like domain. Because *S*-nitrosylation involves the transfer of NO^+ group

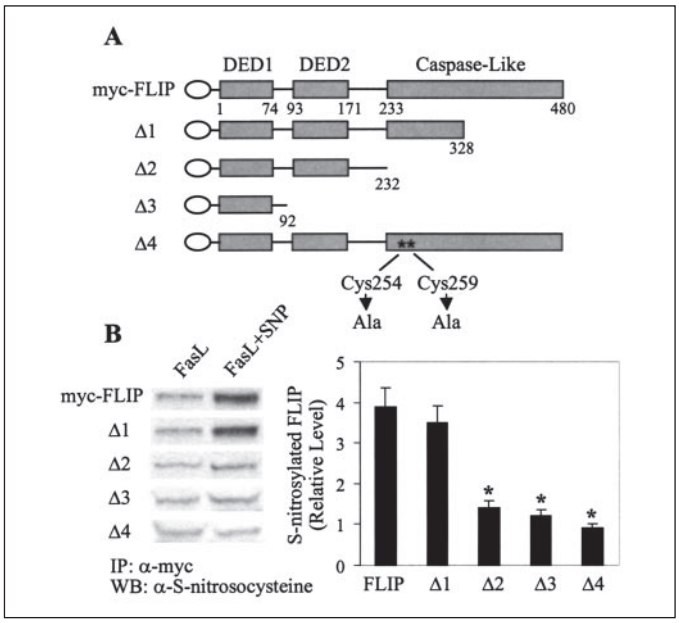

FIGURE 6. **The caspase-like domain of FLIP is required for *S*-nitrosylation.** *A*, schematic structures of FLIP and various constructs (Δ1-Δ4) used in this study. *DED* stands for death effector domain. Amino acids present in each construct are labeled. *Asterisks* indicate Cys-254 and Cys-259 to alanine mutations in the caspase-like domain. *B*, *S*-nitrosylation of FLIP and its mutants were analyzed by transient transfection and immunoprecipitation (*IP*) with Myc antibody as described in Fig. 5. The density of *S*-nitrosylated bands was determined by densitometry and normalized against FasL-treated control bands. Plots are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* myc-FLIP-transfected control. *WB*, Western blot.

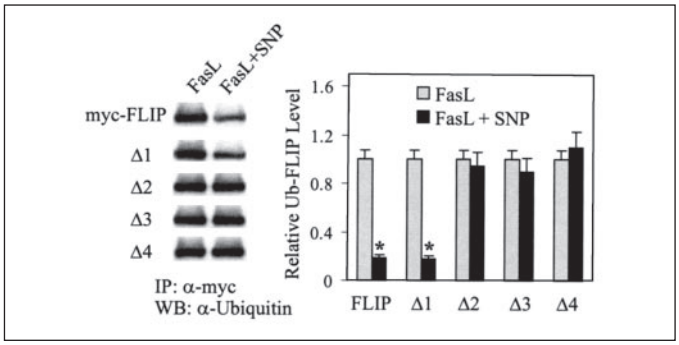

FIGURE 7. ***S*-Nitrosylation inhibits ubiquitination of FLIP by FasL.** BEAS cells were transiently transfected with ubiquitin and Myc-FLIP or its mutant plasmids. One day after the transfection, cells were treated with FasL (100 ng/ml) in the presence or absence of SNP (300 μg/ml) for 2 h, and cell lysates were prepared for immunoprecipitation using Myc antibody. The immunoprecipitated (*IP*) proteins were analyzed by Western blot (*WB*) with antibody against ubiquitin. Band densities were normalized against FasL-treated controls. Plots are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* FasL-treated controls.

to an active site on cysteine residues, we examined the presence of these residues in the target region and found two at position 254 and 259. We mutated the two residues (Δ4) to determine whether the mutations interfere with the *S*-nitrosylation of FLIP. As shown in Fig. 6*B*, mutations of these cysteine residues (to alanine) resulted in a complete inhibition of FLIP nitrosylation. Fig. 7 also shows that such mutations effectively inhibited the protective effect of NO on FLIP ubiquitination, supporting the role of *S*-nitrosylation in the ubiquitination process and demonstrating the requirement of Cys-254 and Cys-259 in FLIP nitrosylation. Mutation of either one of the cysteine residues had only a partial effect on *S*-nitrosylation of FLIP (result not shown), indicating that both residues are required for effective nitrosylation of the FLIP protein.

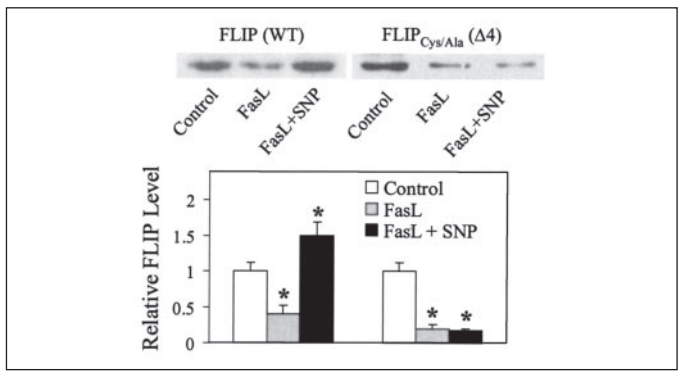

FIGURE 8. ***S*-Nitrosylation of Cys-254 and Cys-259 inhibits FLIP degradation induced by FasL.** BEAS cells were transiently transfected with myc-FLIP or Δ4 mutant plasmid and then treated 1 day later with FasL (100 ng/ml) in the presence or absence of SNP (300 μg/ml) for 12 h. Lysates were immunoprecipitated with Myc antibody and analyzed by Western blot using antibody against FLIP. Band densities were normalized against non-treated controls. Plots are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* non-treated controls.

Mutation of Cys-254 and Cys-259 Protects FasL-induced FLIP Degradation—Inhibition of FLIP ubiquitination by NO should lead to a decrease in proteasomal degradation of FLIP, and likewise, failure to inhibit this ubiquitination in the FLIP Cys/Ala mutant should lead to its increased degradation. As expected, our results show that the NO donor SNP was able to prevent FLIP degradation by FasL as indicated by its increased expression level (Fig. 8). On the other hand, the NO donor had no protective effect on the FLIP mutant, suggesting that *S*-nitrosylation of FLIP, in addition to preventing ubiquitination, also protects this molecule from FasL-induced proteasomal degradation.

DISCUSSION

The anti-apoptotic 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 (for review, see Ref. 43). Although the importance of gene expression in regulating apoptotic signal transduction has been emphasized in numerous studies, post-translational modifications such as ubiquitination and phosphorylation have recently emerged as important regulators of proteins in the death receptor pathway (for reviews, see Refs. 44 and 45). Ubiquitin-mediated degradation of FLIP by the proteasome has been implicated under different conditions, including peroxisome proliferator-activated receptor ligand and p53 activation, chemotherapeutic administration, and viral infection (37–40). However, the mechanisms underlying this regulation and in particular those relevant to Fas signaling have not been demonstrated. Our results showed that treatment of the cells with FasL resulted in a rapid ubiquitination and proteasomal degradation of FLIP with a concomitant increase in caspase-8 activation and apoptosis. For proteins whose levels are regulated by ubiquitin-proteasomal degradation, ubiquitination is induced by binding of the proteins to E3 ubiquitin ligases (for review, see Ref. 46). In this regard, FLIP has been shown to bind and activate tumor necrosis factor receptor-associated factor (TRAF)-2, which contains a RING finger domain known to possess E3 ligase activity (47, 48). It is, therefore, conceivable that during Fas stimulation TRAF-2 is recruited to the death signaling complex along with FLIP and exerts its ligase activity leading to FLIP ubiquitination and degradation. Several other mechanisms, including up-regulation of TRAF-2 or other types of FLIP-binding E3 ubiquitin ligases, could also be envisioned.

Increasing evidence show that NO plays an important role in regulating many key proteins in the death receptor as well as mitochondrial pathway of apoptosis. Although it is more widely accepted that mito-

chondrial depolarization and activation of caspase-3 play a major role in NO regulation of apoptosis (49, 50), recent studies show that NO is also involved in death receptor signaling (29, 31, 50). It is suggested that the anti-apoptotic effect of NO can be mediated through a number of mechanisms such as nitrosylation and inactivation of caspases, up-regulation of p53, and heat shock proteins (51). In Fas-mediated apoptosis, NO has been reported to have anti-apoptotic effect (29, 52), but its underlying mechanisms of regulation are not well characterized. Consistent with previous reports in other cell systems (29, 52), our results showed that NO was able to inhibit FasL-induced apoptosis in epithelial BEAS cells. We further showed that FasL treatment induced down-regulation of FLIP without significant effect on Fas or FADD expression levels. Co-administration of FasL with the NO donors, SNP or DPTA NONOate, attenuated the apoptotic effect of FasL, whereas the NO inhibitor AG or PTIO promoted it, further supporting the anti-apoptotic role of NO in the Fas signaling process.

NO has previously been shown to prevent apoptosis induced by chemotherapeutic agents through complex mechanisms that involve mitochondrial signaling and cGMP/protein kinase G-mediated up-regulation of Bcl-2 (24, 28, 53–55). However, in most cases the protection of NO against apoptosis is independent of cGMP, suggesting alternate pathways of regulation. Furthermore, the mechanisms by which NO regulates apoptosis in the death receptor pathway have not been clearly elucidated. Although NO can up-regulate other anti-apoptotic proteins such as heme oxygenase-1 and metallothionein (56, 57), the most effective way of NO-mediated inhibition of apoptosis is *S*-nitrosylation of key apoptotic proteins (41, 42). In this study we demonstrated that NO can nitrosylate FLIP and prevent its degradation via the ubiquitin-proteasome pathway. Gene deletion analysis revealed the importance of the caspase-like domain of FLIP in the nitrosylation process. Mutational analysis further showed that Cys-254 and Cys-259 of FLIP were responsible for its nitrosylation. Although *S*-nitrosylation of cysteine residues was shown to be important in preventing FLIP ubiquitination, it is not absolutely required for this inhibition since FLIP lacking the two cysteine residues ($\Delta 2$ and $\Delta 3$) can still be ubiquitinated to some degree, indicating that the ubiquitination and *S*-nitrosylation processes are functionally separated and differently regulated by NO.

It has been suggested that NO might inhibit Fas-mediated apoptosis through a decrease in Fas expression (58). However, our Western blot analysis failed to detect the inhibitory effect of SNP on Fas expression at the concentrations that inhibited FasL-induced apoptosis. A similar finding was also observed in granulosa cells (29), although FLIP expression was not detected in that study. The likely explanation for the observed discrepancy may be the difference in experimental design and cell type used. It might be possible that other mechanisms of NO regulation such as induction of heat shock proteins (59), up-regulation of Bcl-2 (58), and suppression of Bax expression (59) may also be involved in this process. The established importance of FLIP in death receptor signaling, however, supports the role of this molecule and its regulation by NO in FasL-induced apoptosis.

Although FLIP has been perceived primarily as an inhibitor of apoptosis, increasing evidence also suggest that this protein plays an additional role in cell survival and proliferation. For examples, overexpression of FLIP has been shown to activate NF-κB (60–62), and inhibition of this pathway by dominant expression of its inhibitory subunit IκB decreased cell survival (63). FLIP has also been shown to activate NF-κB through its ability to recruit key adapter proteins such as TRAF-2 and receptor-interacting protein-1 to the death signaling complex (47, 62). Because TRAF-2 may have a role in ubiquitination of FLIP as earlier described and since NO can modulate this ubiquitination process, it is likely that NO may also play a role in cell survival regulation through NF-κB signaling. Thus, NO may be a key regulator of death and survival in the death receptor pathway, and it cannot only determine whether the apoptosis pathway is turned on or off but also allows the cell to switch between cell death and survival.

In conclusion, our data provide evidence that FasL can induce down-regulation of FLIP through proteasome-mediated degradation. NO negatively regulates this process through its ability to inhibit ubiquitination. It is also worth noting that this regulation occurs via *S*-nitrosylation of FLIP, which interferes with the ubiquitination process. This novel function of NO in the death receptor pathway of apoptosis may have important implications in cell death resistance and pathogenesis of related apoptosis disorders.

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