Nitrosative stress produced by cytokines predisposes to apoptotic cell death. However, the molecular mechanism by which this occurs is not well understood. We have shown previously that nitric oxide (NO) regulates the activity of the anti-apoptotic transcription factor NF-κB. Here we demonstrate that the inhibition of NF-κB by NO sensitizes A549 and Jurkat T cells to tumor necrosis factor-α (TNFα)-induced apoptosis. The molecular basis of NF-κB inhibition is different in the two cell types. In A549 cells, NO functions at the nuclear level to inhibit NF-κB by S-nitrosylation. In Jurkat cells, NO inhibits the NF-κB activating pathway in the cytoplasm at a step proximal to the degradation of IκBα. The inhibition of NF-κB is reflected in the level of intracellular S-nitrosothiols, which are constitutively metabolized. These data suggest that NO can influence cell death by modulating NF-κB activity with the sites of inhibition being cell type-specific. The data also show that NO bioactivity regulates tumor necrosis factor-α signaling.

NF-κB is a transcriptional activator of genes involved in inflammation, including numerous cytokines, cytokine receptors, and adhesion molecules (1). Activation of NF-κB also up-regulates genes that protect cells from apoptosis induced by TNFα (2) (and other stimuli) (2–4). The NF-κB-dependent anti-apoptotic mechanism(s) is incompletely understood. Recent evidence suggests that the inhibition of c-Jun N-terminal kinase mitogen-activated protein (JNK MAP) pathway is involved (5, 6). In addition, NF-κB has been shown to express the inhibition of anti-apoptotic proteins c-IAP1, c-IAP2, TRAF2, and Bel-2 family members (7–10).

NF-κB transcription is sensitive to oxidative and nitrosative stress (11). An oxidizing cytoplasmic environment is typically associated with NF-κB activation, yet oxidation or nitrosation of the NF-κB heterodimer (p50-p65) prevents DNA binding (12–15). These data suggest that NO/redox exerts control over NF-κB at multiple loci within the signal transduction pathways that transmit inflammatory signals from the plasma membrane to the nucleus. p21^WAF1, JNK kinase, and the p50 monomer (of p50-p65) have been identified as sites of S-nitrosylation that mediate the stimulation or inhibition of NF-κB by NO (15–18). Additional molecular mechanisms by which NO can inhibit NF-κB (e.g. the effect on IκB kinase(s)) also exist (19). The effect of NO on NF-κB thus depends upon the activating stimulus or signal, cell type, and local redox milieu (11).

NO is a known regulator of both pro- and anti-apoptotic pathways. For instance, NO functions as an anti-apoptotic mediator by inhibiting caspase-3 activation via nitrosylation of the procaspase-3 precursor (20). With an appropriate cellular stimulus (i.e. Fas ligation), denitrosylation of procaspase-3 occurs, thereby activating caspase-3 and initiating cellular apoptosis. Alternatively, NO may stimulate apoptosis by increasing cellular p53 levels (via an effect on proteosomal degradation) (21), by activating cytochrome c (22), or perhaps by opening the mitochondrial permeability transition pore (23).

Previously, we have described an apoptotic model of nitrosative stress, which is produced by cytokine up-regulation of NOS2 (24), and we have also reported that NO inhibits NF-κB in cells by S-nitrosylation (17). In the present study, we sought to determine whether NO inhibition of NF-κB by S-nitrosylation contributes to apoptosis. Using two different human cell lines (A549 respiratory epithelial and Jurkat T cells), we demonstrate that nitrosative stress sensitizes cells to TNFα-mediated apoptosis and provide evidence for the role of NF-κB inhibition in this process. We also show that the mechanism of NF-κB inhibition is, in fact, different in the two cell lines. In A549 cells, NO functions at the nuclear level by inhibiting NF-κB DNA binding through a reversible, redox-based modification of the p50-p65 heterodimer (consistent with S-nitrosylation). In Jurkat T cells, NO functions in the cytoplasm by inhibiting IκBα degradation, thus preventing the nuclear translocation of NF-κB.

MATERIALS AND METHODS

Cell Lines and Plasmids—A549 (CCL-185) and Jurkat T cells were obtained from ATCC (Manassas, VA). A549 cells were grown in F12K media supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Jurkat T cells were grown in Dulbecco’s modified Eagle’s medium with high glucose supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cultures were maintained in 95% air, 5% CO₂ at 37 °C. The pNFB-Luc and pRL-CMV (RL, Renilla luciferase) plasmids were purchased from Stratagene and Promega, respectively.

Apoptosis Assays—Apoptotic cells were determined using a modified annexin V protocol (Molecular Probes). Jurkat cells (2 × 10⁶ cells) or A549 cells (50–75% confluent) were grown in 6-well tissue culture plates. After the indicated treatments, cells were harvested, washed with cold PBS, and resuspended in 0.5 ml of 1× binding buffer. 5 μl each of annexin V-PE and 7-aminocoumarin were added, and the cells were incubated at 25 °C for 15 min in the dark. The cells were analyzed for annexin V-PE and 7-aminocoumarin fluorescence by FACS (FACS Calibur, Becton Dickinson) analysis using standard filters.

NF-κB Reporter Assay—A549 cells were grown to ~75% confluence in 6-well plates. Cells were washed with serum-free media and transfected with 1 μg of pNFB-Luc and 0.5 μg of pRL-CMV using SuperFect filters.

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† The abbreviations used are: TNFα, tumor necrosis factor-α; NO, nitric oxide; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; IKK, IκB kinase complex; Lux, luciferase; RL, Renilla luciferase; PBS, phosphate-buffered saline; DTT, 1,4-dithiothreitol; FACS, fluorescence-activated cell sorter; DETA, diethylenetriamine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1; CSNO, S-nitrosocysteine; SNO, S-nitrosothiol; 8-Br-cGMP, 8-Bromoguanosine 3′,5′-cyclic monophosphate.

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The nuclear pellet was reconstituted in 15 ml of Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.5 mM EDTA, 1.5 mM MgCl₂, 0.2% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride), and the cell suspension was placed on ice for 10 min to allow for lysis. Nuclei were pelleted by centrifugation at 3500 g for 10 min at 4 °C, and the supernatant (cytoplasmic extract) was collected. The nuclear pellet was reconstituted in 15 ml of Buffer C (20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, and 5% glycerol) with 1 µM DTT, 0.5 mM phenylmethylsulfonyl fluoride) and placed on a rocking platform at 4 °C for 30 min. The nuclear lysate was then clarified by centrifugation at 14,000 × g for 20 min, and the supernatant was collected (nuclear extract).

Nuclear extracts were diluted with 40 µl of Buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride). The protein concentration of the extracts was determined using the BCA method (Pierce), and extracts were used immediately or stored at −80 °C. Electrophoretic Mobility Shift Assay—Eight µg of nuclear protein was used per reaction. Reaction buffer contained 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, and 5% glycerol with 1 µg of p50/p65, 1 µg of p65, and 10 µg of bovine serum albumin. The NF-κB consensus oligonucleotide (5'-AGTGTAGGAGGACTTTCCAGGCGG-3') (Promega) was end-labeled with [γ-32P]ATP (PerkinElmer Life Sciences) using T4 kinase. 1 µl of radiolabeled NF-κB probe (activity 30,000–80,000 cpm/µl) was added to the reaction mixture, and the samples were incubated for 20 min at room temperature. The reaction was terminated by the addition of 6 µl of a 15% Ficoll solution with indicator dyes. Samples loaded on a 5% non-denaturing polyacrylamide gel in 1× Tris borate EDTA (TBE) buffer and run at 200 V for 2 h. Gels were transferred to Whattman No. 2MM filter paper, vacuum-dried at 80 °C for 2 h, and exposed to Kodak XOMAT film for 2–4 h at −80 °C.

Immunofluorescent Staining—A549 cells were seeded onto two-well chamber slides and grown to ~50% confluence in complete media. After the indicated treatments, cells were washed with cold PBS and fixed with ice-cold methanol (−20 °C) for 5 min. Jurkat cells were fixed to slides using a cytospin (600 × g for 6 min) after the indicated treatments. The cells were then fixed in 75% ethanol at −20 °C overnight.

Prior to immunostaining, both A549 and Jurkat cell slides were rinsed with PBS and blocked with 10% goat serum for 2 h at 25 °C. Cells were then rinsed with PBS and incubated with a rabbit polyclonal antibody against NF-κB p65 subunit (Santa Cruz Biotechnology) for 1 h at 25 °C. Immunoreactivity with the primary antibody was detected using an fluorescein isothiocyanate-conjugated goat anti-rabbit anti-sense (Santa Cruz Biotechnology). Cells were mounted in antifade solution and visualized with a confocal microscope (Zeiss 510 confocal laser scanning).

Immunoblot Analysis—Equal amounts (40–60 µg) of cytoplasmic proteins were separated by SDS-PAGE on a 4–20% Tris-glycine gel. After transferring to nitrocellulose, blots were probed with a rabbit polyclonal antibody to IκBα (Santa Cruz Biotechnology) at a 1:500 dilution. Immunoreactivity was visualized with enhanced chemiluminescence (Bio-Rad).

RESULTS

NO Inhibits NF-κB-dependent Transcription by TNFα—Treatment with 1 mM S-nitrosocysteine (CSNO) or 1 mM DETA-NONOate (NONOate) significantly decreased TNFα-stimulated NF-κB activity in A549 cells (Fig. 1A). NO also attenuated NF-κB activation in TNFα-stimulated Jurkat cells, albeit at a slightly lower NO concentration (0.5 mM CSNO or NONOate) (Fig. 1B). Thus, the inhibition of NF-κB by NO is likely to be a generalized phenomenon. Lower concentrations of NO had no effect on TNFα-induced NF-κB activity, nor did NO alone alter basal NF-κB activity in either cell type (data not shown). The fact that Jurkat cells are more sensitive to NO then A549 cells raises the possibility that different mechanisms of NF-κB inhibition operate in the two cell lines.

NO Induces Apoptosis in TNFα-stimulated Cells—We sought to determine whether apoptosis is influenced by NO in A549 and Jurkat cells. Using an annexin V assay, we showed that the amount of apoptosis (percentage of annexin V + cells) did not change in either cell line after TNFα or NO (CSNO or NONOate) treatment alone (Fig. 2). However, the combination of TNFα and NO (at concentrations required to inhibit NF-κB) significantly increased apoptosis in both cell types (Fig. 2). In both cell lines, co-treatment with CSNO induced apoptosis to a greater degree than NONOate. In keeping with the lack of effect on NF-κB activity, lower concentrations of NO (10 or 100 µM) did not alter apoptosis in TNFα-stimulated cells.

NO Augmentation of Apoptosis in TNFα-stimulated Cells Is Analogous to the Effects of Transcriptional and NF-κB Inhibitors—Transcriptional inhibitors are known to sensitize cells to TNFα-induced apoptosis in part by preventing NF-κB-dependent up-regulation of anti-apoptotic genes (26). To provide further evidence that the mechanism for NO-induced apoptosis in TNFα-stimulated cells is NF-κB dependent, we measured the effects of the transcription inhibitor actinomycin D with NO in TNFα-stimulated A549 and Jurkat cells. Treatment with TNFα and actinomycin D (0.5 µM) significantly increased apoptotic death in both cell lines (as compared with TNFα or actinomycin D alone) with the amount of apoptosis similar to that seen with TNFα and NO (Fig. 3). Proteasome inhibitors prevent the degradation of IκBα and thereby provide a more specific means of blocking NF-κB tran-
Similar to NO, treatment of TNFα-stimulated Jurkat cells with the proteasome inhibitor lactacystin both inhibited NF-κB activation (by ~85%) and significantly increased apoptosis (Fig. 3). That neither lactacystin nor another proteasome inhibitor, MG-130, inhibited NF-κB-dependent transcription or induced apoptosis in TNFα-stimulated A549 cells (data not shown) provides added assurance that the apoptosis-inducing effects of these compounds reflect NF-κB inhibition.

Intracellular S-Nitrosothiol (SNO) Levels and the Induction of Apoptosis—An increase in endogenous NOS2 activity and NO production, which is dependent on NF-κB, has been shown to promote apoptosis in macrophages (24). The increase in apoptotic death correlated with a marked rise in intracellular SNOs. However, the response to NOS2 activity was quite different in A549 cells. After 16 h of cytokine stimulation, A549 cells demonstrated a marked increase in NOS2 expression and activity (as measured by NOx production) (data not shown) but no increase in apoptosis (control 3.8 ± 0.1 versus cytokine-stimulated 4.1 ± 1.0%) and only a modest rise in intracellular SNOs (Fig. 4). This level of nitrosative stress is not adequate to induce apoptosis directly (24) and thus corroborate the lack of effect of NOS inhibitors on NF-κB-dependent transcription in cytokine-stimulated A549 cells (data not shown). On the other hand, treatment of A549 cells with exogenous NO (1 mM CSNO or NONOate) increased intracellular SNOs ~10-fold (Fig. 4), analogous to levels required for apoptosis in lipopolysaccharide-stimulated macrophages (24). Lower concentrations of exogenous NO did not increase intracellular SNO above baseline,
nor did co-treatment with TNFα alter SNO levels (data not shown). These data indicate that the requirement for high amounts of exogenous NO to promote apoptosis is well rationalized by the inability of lower concentrations (even 0.1 mM) to produce a nitrosative stress.

**NO-induced Apoptosis in TNFα-stimulated Cells Is cGMP- and Caspase-3-independent**—Activation of downstream caspases has been implicated in both NO- and TNFα-mediated apoptosis (6, 28). Moreover, we have shown that Fas-induced activation of caspase-3 is coupled to denitrosylation of procaspase-3 in lymphocytes (20). Consistent with these data, Jurkat T cells showed a modest increase in caspase-3 activity after TNFα stimulation (analogous to Fas ligation) (Fig. 5A). However, the TNFα-induced increase in caspase-3 activity was not affected by concurrent CSNO treatment. Further, caspase-3 activity was not detectable in A549 cells following treatment with TNFα, CSNO, TNFα and CSNO, or Fas ligation (data not shown). These data imply that the mechanism by which NO induces apoptosis in TNFα-stimulated cells appears to be largely independent of caspase-3.

NO can sensitize cells to apoptosis by both cyclic GMP-dependent and -independent mechanisms (29). To investigate whether the induction of apoptosis by TNFα and NO in Jurkat T and A549 cells is cyclic GMP-mediated, the cells were cotreated with TNFα and the cyclic GMP analogue, 8-Br-cGMP (100 μM). No increase in apoptosis was seen in either cell line under these conditions (Fig. 5B). Moreover, there was no attenuation in TNFα- and CSNO-induced apoptosis in the presence of the guanylate cyclase inhibitor ODQ (10 μM) (Fig. 5B). These results argue against a role for cGMP in the induction of apoptosis by NO in TNFα-stimulated cells.

**NO Inhibits NF-κB in Jurkat T and A549 Cells by Two Different Mechanisms**—We and others have shown that NO can inhibit NF-κB by nitrosylation of a critical cysteine on the p50 subunit of the p50-p65 heterodimer (15, 17, 30). Nitrosylation of NF-κB (p50-p65) functions at the nuclear level and is reversible by reduction of the nitrosylated cysteine. This inhibitory mechanism appears to predominate in A549 cells as the NO-induced decrease in NF-κB DNA binding (in TNFα-stimulated cells) is reversed by treatment of nuclear protein extracts with the reducing agent DTT (Fig. 6A). Furthermore, nuclear translocation of NF-κB is not inhibited (as demonstrated by p65 immunofluorescent staining) nor is IκBα degradation affected after NO treatment of TNFα-stimulated A549 cells, indicating that inhibition is occurring at the nuclear level (Fig. 6, B and C).

**Discussion**

Nitrosative stress has been causally linked to apoptosis, but the molecular details are not well understood. The results of this study indicate that the inhibition of the anti-apoptotic transcription factor, NF-κB, is one mechanism by which NO can initiate apoptosis. NO does not, however, induce apoptosis alone. Rather, it works in concert with TNFα to relieve the inhibitory effects of NF-κB. The inhibition of NF-κB has been shown previously to promote TNFα-induced cell death (2–4), but the inhibition was achieved through nonphysiological interventions. This study is the first to link a physiological reg-
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The mechanism of NO inhibition of NF-κB activity (that is, NO bioactivity) with the NF-κB-dependent control of apoptosis.

The existence of multiple redox-sensitive steps in the NF-κB activating pathway has been described previously. In the nucleus, NF-κB DNA binding can only occur if a target cytokine (cytome 62) on the p50 monomer is reduced (31). Glutathiolation or nitrosylation of this residue has been demonstrated to inhibit NF-κB DNA binding in vitro (17, 32). On the other hand, an oxidative environment in the cytoplasm can activate NF-κB through effects on the IkB kinase complex (IkB) (33, 34). Oxidative activation of NF-κB is, however, cell- and stimulus-specific (14, 35). If oxidation of a target cytokine in the IKK complex is required for activation in Jurkat cells, nitrosylation of this same cytokine might prevent activation. Indeed, there is recent precedent for an oxidative modification in the transcriptional activator OxyR eliciting a response different from nitrosylation (36). Alternatively, different components of the IKK complex may be targeted for posttranslational modification by oxidation and nitrosylation, respectively.

Induction of NOS2 is NF-κB-dependent (37). One might therefore envision a feedback loop whereby NO down-regulates its own synthesis. However, NO inhibition had no effect on NF-κB-dependent transcription in the NOS2-stimulated cells (data not shown), and although exogenous NO donors initiated apoptosis in TNFa-stimulated A549 cells, the cytokine-induced increase in NOS2 activity did not. An explanation for these data can be found in the levels of intracellular SNOs (a surrogate marker of nitrosative stress) under the various conditions. In the NOS2-stimulated cells, SNOs were only 3-fold higher than in controls. In previous studies, we determined that at least 10-fold increases in SNO were necessary to initiate apoptosis (24), a level achieved in A549 cells by treatment with 1 mM NO donor (CSNO or NONOate). The more modest rise in intracellular SNOs with NOS2 stimulation occurred despite a 20-fold increase in cellular NO production. In addition, 0.1 mM NO donor did not increase intracellular SNOs. These results indicate that intracellular SNOs are actively metabolized and are furthermore compatible with the emerging evidence that SNO levels are precisely controlled with a balance between production and breakdown (or cellular export). Cell-specific differences in the activity of nitrosative defense enzymes might explain the varying susceptibility of cells to NO-induced apoptosis. In particular, GSNO reductase, a recently described SNO-metabolizing enzyme, is very active in A549 cells (38).

In summary, we demonstrate that NO sensitizes cells to TNFa-induced apoptosis. The mechanism for this sensitization is consistent with the inhibition of the anti-apoptotic transcription factor NF-κB. In addition, we show that at least two targets for NO inhibition of the NF-κB activating pathway exist, one in the cytoplasm (possibly the IKK complex) and the other in the nucleus (p50-p65). Finally, our data expand on the link between the TNF receptor family and NO signaling in the context of apoptosis.

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