Inhibition of NF-κB by S-Nitrosylation

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ABSTRACT: It is not clear if redox regulation of transcription is the consequence of direct redox-related modifications of transcription factors, or if it occurs at some other redox-sensitive step. One obstacle has been the inability to demonstrate redox-related modifications of transcription factors in vivo. The redox-sensitive transcriptional activator NF-κB (p50−p65) is a case in point. Its activity in vitro can be inhibited by S-nitrosylation of a critical thiol in the DNA-interacting p50 subunit, but modulation of NF-κB activity by nitric oxide synthase (NOS) has been attributed to other mechanisms. Herein we show that cellular NF-κB activity is in fact regulated by S-nitrosylation. We observed that both *S*-nitrosocysteine and cytokine-activated NOS2 inhibited NF-κB in human respiratory cells or murine macrophages. This inhibition was reversed by addition of the denitrosylating agent dithiothreitol to cellular extracts, whereas NO bioactivity did not affect the TNFα-induced degradation of IκBα or the nuclear translocation of p65. Recapitulation of these conditions in vitro resulted in S-nitrosylation of recombinant p50, thereby inhibiting its binding to DNA, and this effect was reversed by dithiothreitol. Further, an increase in S-nitrosylated p50 was detected in cells, and the level was modulated by TNFα. Taken together, these data suggest that S-nitrosylation of p50 is a physiological mechanism of NF-κB regulation.

NF-κB is a transcription factor that regulates numerous genes active in the inflammatory response including IL-8, TNFα, MCP-1, ICAM-1, and NOS[1] 2 (*1*). NF-κB is a dimeric protein comprised of any of at least five different members of the Rel family [p50, p65 (RelA), p52, c-Rel, and RelB] (*2*). The primary transcriptional-activating form of NF-κB is the p50−p65 heterodimer. In its inactive state, NF-κB is sequestered in the cytoplasm bound to an IκB inhibitory protein (i.e., IκBα, IκB*β*, IκB*ϵ*). With an appropriate stimulus (e.g., TNFα, UV light, H_2O_2), an intracellular kinase cascade is triggered that results in the phosphorylation, ubiquitination, and proteosomal degradation of the IκB proteins (*2*). These events allow NF-κB to translocate to the nucleus, bind to target DNA sites, and activate gene transcription.

NF-κB is one of several transcription factors (including AP-1, Pax-8, GABPα) that display redox-sensitive DNA binding (*3*−*5*). That is, these transcription factors require a reducing environment to bind DNA. In the case of AP-1 and NF-κB, this redox sensitivity is conferred by a single cysteine residue within the DNA binding site (*3*, *6*). An oxidative modification of these thiols inhibits DNA binding in vitro (*7*). These cysteines are also potential sites for posttranslational modification by S-nitrosylation (*8*, *9*). It is unclear, however, if such modifications (that is, nitrosylation or oxidation) are relevant mechanisms of regulation in vivo (*10*).

Cellular production of NO is controlled, in part, by NF-κB activation (*11*). In particular, deletion of κB sites from the NOS2 promoter prevents the cytokine-induced increase in NOS2 transcription (*12*). NO or related molecules, in turn, have been found to modulate NF-κB activity in the cell. It has been reported, moreover, that NO can both stimulate and inhibit NF-κB activity (*13*−*15*). NO activation of NF-κB results from stimulation of p21 ras (*13*); that is, the NO effect is upstream of NF-κB, whereas the mechanism(s) of NO inhibition is (are) controversial.

It was originally suggested that NO stabilizes the IκBα cytoplasmic complex and increases IκBα transcription, thereby decreasing NF-κB activation (*14*, *16*). These studies reported that *S*-nitrosothiols (SNOs) had no direct effect on the transcription factor. However, later work has shown that p50 can be S-nitrosylated in vitro, thereby attenuating NF-κB DNA binding (*8*, *9*). The reasons for these discrepancies are unclear and a consensus on NO regulation of NF-κB has not emerged. In this study, we provide evidence that S-nitrosylation inhibits NF-κB in vivo, that this inhibition occurs within the nucleus, and that the pathway may be controlled by TNFα.

EXPERIMENTAL PROCEDURES

Cell Lines and Plasmids. A549 (CCL-185), RAW 264.7 (TIB-71), and 293 (CRL-1573) cells were obtained from ATCC (Rockville, MD). The p3×κBluc and p50CMV plasmids were kind gifts from Dr. Albert Baldwin Jr. (University of North Carolina, Chapel Hill, NC). pSV-*β*-galactosidase was purchased from Promega.

Cell Culture. A549 cells were grown in F12K media supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. RAW 264.7

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[1] Abbreviations: CSNO, *S*-nitrosocysteine; DTT, 1,4-dithiothreitol; EMSA, electrophoretic mobility shift assay; IP, immunoprecipitate; NO, nitric oxide; NOS, nitric oxide synthase; SNO, *S*-nitrosothiol.

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and 293 cells were grown in DMEM with high glucose supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cultures were maintained in 95% air, 5% CO_2 at 37 °C.

Cytoplasmic and Nuclear Extracts. Preparation of cytoplasmic and nuclear extracts was done according to Osborn (*17*) with minor modifications. Extracts were prepared with or without the addition of 1,4-dithiothreitol (DTT) (1 mM) to extraction buffers. Specifically, cells were grown to near-confluence in 10 cm culture plates. After the indicated treatments, cells were washed once in cold PBS and harvested by gentle scraping. The cells were then pelleted by centrifugation and rapidly frozen in a dry ice/ethanol bath, the frozen pellet was resuspended in 75 μL of Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.5 mM EDTA, 1.5 mM $MgCl_2$, 0.2% NP-40, and 0.5 mM PMSF), 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 μL of Buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 25% glycerol, and 0.5 mM PMSF) and placed on a rocking platform at 4 °C for 30 min. The nuclear lysate was then clarified by centrifugation at 14000*g* for 20 min and the supernatant 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 PMSF). The protein concentration of the extracts was determined with the Coomassie Plus reagent (Pierce), and the extracts were used immediately or stored at −80 °C.

Electrophoretic Mobility Shift Assay (EMSA). Eight micrograms 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 pdI·pdC, 1 μg of $pd(N)_6$, and 10 μg of bovine serum albumin. The NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Promega) was end-labeled with [γ-^{32}P]ATP (NEN Life Science Products) using T4 kinase. One microliter 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. For supershift experiments, 1 μL of anti-p50 or anti-p65 antibodies (Santa Cruz Biotechnology) was added to the reaction mixture 30 min prior to the addition of the oligonucleotide probe. Samples were loaded onto a 5% nondenaturing polyacrylamide gel in 1× Tris−borate EDTA (TBE) buffer and run at 200 V for 2 h. Gels were transferred to Whatman 2 MM filter paper, vacuum-dried at 80 °C for 2 h, and exposed to Kodak XOMAT film for 2−4 h at −80 °C. Bands were quantified using laser densitometry.

Binding of recombinant p50 (Promega) to the NF-κB oligonucleotide probe was carried out with 1 pmol of p50 protein in buffer containing 20 mM HEPES, pH 7.9, 50 mM NaCl, 10% glycerol, and 40 μM DTT. The oligonucleotide binding reaction was followed as described above.

Immunoblot Analysis. Equal amounts (30−40 μg) of cytoplasmic, nuclear, or whole cell proteins were separated by SDS−PAGE on a 4−20% Tris−glycine gel. After transferring to nitrocellulose, blots were probed with rabbit polyclonal antibodies to Iκβα, p50, or p65 (Santa Cruz Biotechnology) at a 1:500 dilution. Immunoreactivity was visualized with enhanced chemiluminesence (Bio-Rad).

Plamid Transfection. For reporter assays, A549 and RAW 264.7 cells were seeded at a density of (3−5) × 10^5 cells/well in a 6-well plate and grown in complete media for 24−48 h to achieve approximately 75% confluence. Transfection was done using a mixture of 6 μL of Lipofectamine Plus (Gibco-BRL), 1 μg of p3×κBluc, and 1 μg of pCMVβgal in serum-free media. After 5 h, cells were washed and grown for 48 h in complete media prior to the indicated treatments. Cellular activities of luciferase and β-galactosidase were assessed with standard assays (Promega).

p50 was overexpressed in 293 cells. Cells were grown to a density of approximately 75% in 10 cm culture dishes, and then transfected with 5 μg of p50CMV for 3 h using SuperFect reagent (Qiagen). Following removal of the transfection mixture, cells were grown in complete media for another 48 h before use.

Immunoprecipitation. Whole cell lysates of transfected 293 cells were prepared using 4 °C lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5 mM EDTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM PMSF. After preclearing with 10 μg of normal rabbit IgG and 25 μL of protein G−Sepharose (Amersham Pharmacia Biotech), 10 μg of p50 rabbit polyclonal antibody was added to the lysate. After 1 h, 50 μL of protein G−Sepharose beads was added, and immunoprecipitates (IPs) were allowed to form for 3 h. Beads were then collected by centrifugation and washed extensively with lysis buffer. Antigen−antibody complexes were eluted from the beads with 100 mM glycine buffer (pH 2.5) and kept on ice prior to analysis.

Quantification of Free Thiols in p50. Thiols were quantified by labeling with [^{3}H]iodoacetate. Recombinant p50 protein was initially dialyzed to remove DTT from the storage buffer and then deoxygenated under argon. A 100-fold molar excess of sodium cyanoborohydride, a mild reducing agent, was added, and the mixture was incubated at room temperature for 15 min. [^{3}H]Iodoacetate (NEN Life Sciences Products) was added at 100-fold molar excess over protein, and labeling was carried out at room temperature for 20 min. ^{3}H-labeled protein was precipitated with ice-cold 10% trichloroacetic acid (TCA), pelleted by centrifugation, and washed 3 times with 10% TCA, and the washed pellet was resuspended in HEPES buffer (20 mM, pH 7.9). An aliquot was retained to determine protein concentration, and the ^{3}H content of the remainder of the sample was quantified by scintillation counting. Thiols were also quantified by a modified method of Saville as described below (*18*).

S-Nitrosylation of p50. S-Nitrosylation of recombinant p50 was carried out with dialyzed, sodium cyanoborohydride-reduced protein as described above. First, *S*-nitrosocysteine (CSNO) was prepared by combining equimolar concentrations of sodium nitrite and L-cysteine in 0.5 N HCl; the reaction product was diluted to appropriate concentrations in PBS with 0.5 mM EDTA. p50 protein (in DTT-free, HEPES buffer, 20 mM, pH 7.9) was reacted with a 250-fold molar excess of freshly prepared CSNO at room temperature for 5 min. The mixture was than dialyzed against DTT-free, HEPES buffer to remove CSNO (as verified by photolysis−chemiluminescence). An aliquot of dialyzed protein was retained to determine protein concentration, and

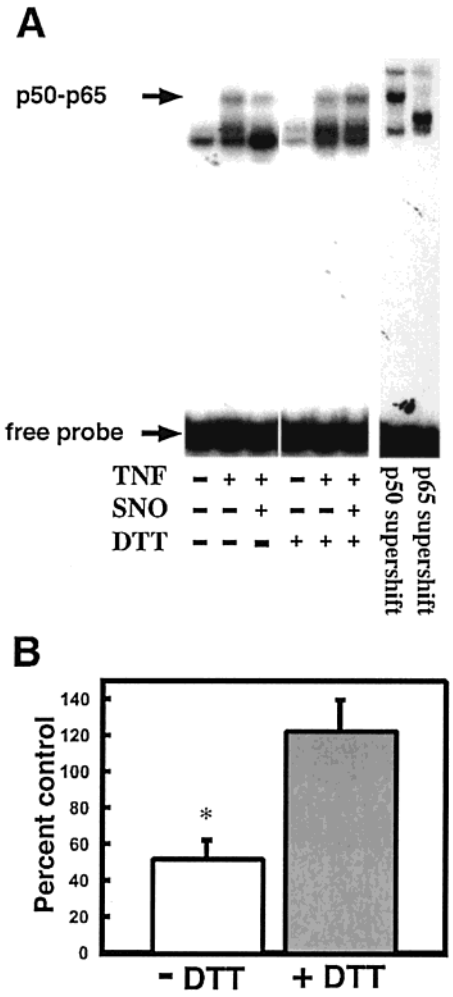

FIGURE 1: Thiol-reversible inhibition of NF-κB by SNO in respiratory epithelial cells. A549 cells were treated with CSNO (1 mM) for 20 min in culture followed by stimulation with TNFα (20 ng/mL) for 30 min. Nuclear extracts were prepared with or without DTT (1 mM) in the extraction buffers. (A) NF-κB DNA binding activity as assessed by EMSA. Antibody supershifting of the NF-κB bands is displayed in the far right panel. Arrows denote the location of the p50−p65 heterodimer and free probe on the gel. The two bands below the p50−p65 heterodimer were constitutively present and were not further characterized. (B) Densitometric quantification of the p50−p65 band on the EMSA. Values are reported as percent control ([TNF + SNO]/TNF alone). ($n = 3$; error bars represent SE.) [*$p < 0.05$ (−DTT compared to +DTT).]

the remainder was analyzed for nitrosothiol content using a photolysis−chemiluminescence method (*18*).

RESULTS

S-Nitrosocysteine Inhibits NF-κB DNA Binding. To examine the effects of SNO on NF-κB activity, A549 cells were pretreated with 1 mM CSNO for 20 min prior to TNFα stimulation. The intracellular concentration of SNO following CSNO treatment was determined to be approximately 1 μM, and no morphological changes or cytotoxic effects [as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay] were seen (data not shown). CSNO treatment decreased TNFα-induced NF-κB activation by ~50% as demonstrated by EMSA (Figure 1A,B). p50 and p65 supershift assays identified the complex that was inhibited as the p50−p65 heterodimer. If DTT (1 mM) was included in the extraction buffers (i.e., added after CSNO exposure), the NO effect on NF-κB activation was reversed. A similar thiol-reversible inhibition of NF-κB by SNO was also seen in RAW 264.7 macrophages stimulated for 30 min with LPS (Figure 2). DTT has been shown to reverse SNO modifications of multiple proteins (*19*−*21*). Taken together, these results are consistent with SNO inhibiting NF-κB via S-nitrosylation of a cysteine within the p50−p65 heterodimer.

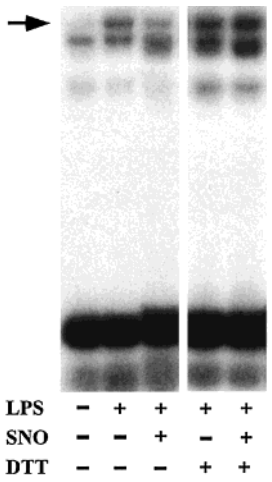

FIGURE 2: Thiol-reversible inhibition of NF-κB by SNO in macrophages. RAW 264.7 cells were treated with CSNO (1 mM) for 20 min in culture followed by stimulation with LPS (500 ng/mL) for 30 min. Nuclear extracts were prepared with or without DTT (1 mM) in the extraction buffers. NF-κB DNA binding activity as assessed by EMSA. Arrow denotes the location of the p50−p65 heterodimer.

NOS Inhibition Increases NF-κB DNA Binding. To determine whether endogenous NO production inhibits NF-κB, A549 cells were stimulated with a cytokine cocktail, which has been shown to maximally induce NOS2 activity in these respiratory epithelial cells (*22*). The effects of NOS inhibition on NF-κB DNA binding were then examined. A marked increase in NF-κB DNA binding was observed if L-NMMA (1 mM) was added prior to cytokine stimulation, but not when the inactive enantiomer D-NMMA (1 mM) was used (Figure 3A,B). This difference in binding was abrogated in the presence of DTT (data not shown). Thus, it appears that endogenous NO production also inhibits NF-κB activity in respiratory epithelial cells by a similar thiol-sensitive mechanism.

NF-κB-Dependent Transcription Is Inhibited by S-Nitrosocysteine. To determine whether SNO also inhibits NF-κB-dependent transcription, NF-κB reporter assays were performed in untreated and CSNO-treated A549 and RAW 264.7 cells. A549 and RAW 264.7 cells were transfected with a NF-κB luciferase construct and stimulated with TNFα (20 ng/mL) or LPS (500 ng/mL), respectively, for 16 h in the presence or absence of CSNO (0.5 mM). SNO treatment significantly decreased NF-κB-dependent luciferase activity in both cell lines (Figure 4A,B). These results confirm that SNO not only inhibits NF-κB DNA binding but also inhibits NF-κB-dependent transcriptional activity in vivo.

S-Nitrosocysteine Does Not Inhibit IκBα Degradation or NF-κB Nuclear Translocation. Prior studies have reported that NO inhibits NF-κB activation through stabilization and increased transcription of the cytoplasmic inhibitory protein IκBα (*14*, *16*). Immunoblot analysis of IκBα was therefore performed to determine whether treatment with CSNO (1

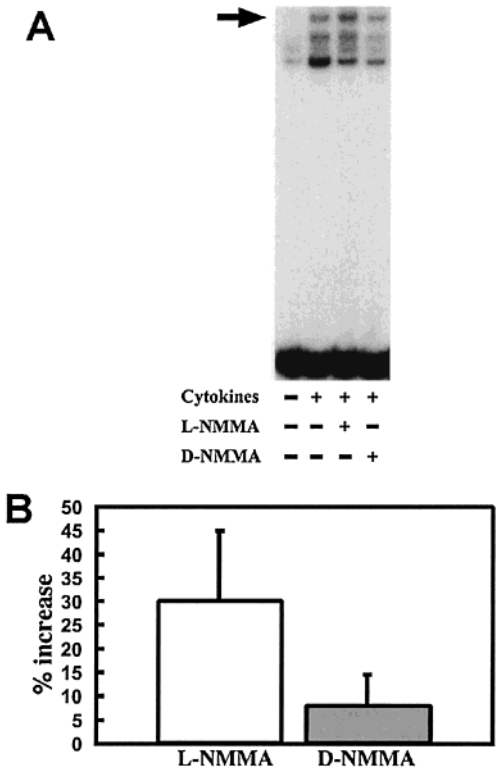

FIGURE 3: NOS inhibition increases NF-κB activity in cytokine-stimulated cells. A549 cells were stimulated with TNFα (20 ng/mL), IL-1β (10 ng/mL), γ-interferon (700 units/mL), and LPS (500 ng/mL) for 8 h in culture. L-NMMA (1 mM) or D-NMMA (1 mM) was added concurrently with the cytokines (A) NF-κB DNA binding activity as assessed by EMSA. The arrow denotes the location of the p50−p65 heterodimer on the gel. (B) Densitometric quantification of the p50−p65 band on the EMSA. Values are reported as percent increase (relative to cytokine stimulation alone). ($n = 3$; error bars represent SE.)

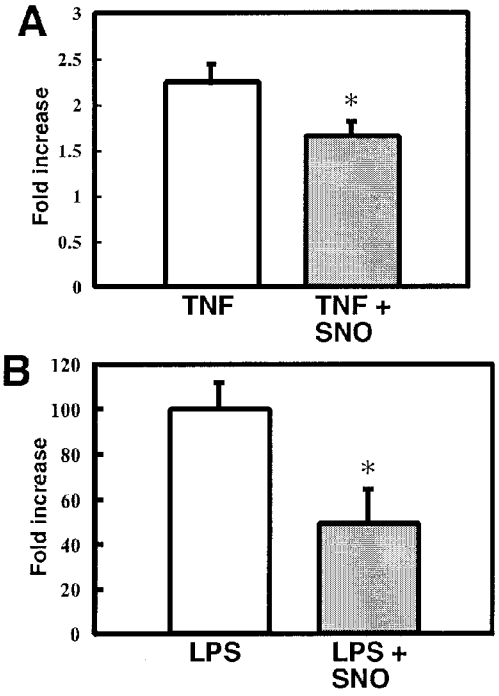

FIGURE 4: SNO inhibits NF-κB-dependent gene transcription. A549 (A) or RAW 264.7 (B) cells were transfected with a NF-κB reporter construct (p3×κBluc) plus pCMVβgal to control for transfection efficiency. 48 h after transfection, cells were stimulated with (A) TNFα (20 ng/mL) or (B) LPS (500 ng/mL) ± SNO (500 μM). Luciferase activity was measured 16 h after stimulation. Results are reported as increase in luciferase activity relative to unstimulated control. ($n = 3$; error bars represent SD.) (*$p < 0.01$.)

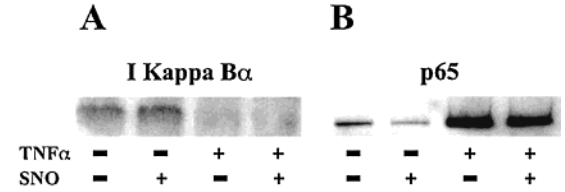

FIGURE 5: SNO does not inhibit IκBα degradation or nuclear translocation of NF-κB in TNFα-stimulated cells. A549 cells were stimulated with TNFα (20 ng/mL) for 20 min. CSNO (SNO, 1 mM) was added 10 min prior to stimulation. Western blots were then performed on equivalent amounts of cytoplasmic (A) or nuclear extracts (B) using antibodies to IκBα or p65, respectively.

mM) affected TNFα-induced degradation of IκBα in A549 cells. SNO did not prevent the cytoplasmic degradation of IκBα in TNFα-stimulated cells (Figure 5A). Moreover, there was no effect of SNO on the increase in nuclear p65 protein levels seen after TNFα stimulation, indicating that nuclear translocation of the NF-κB p50−p65 heterodimer was also not inhibited (Figure 5B). These data indicate that SNO does not inhibit the NF-κB activation pathway proximal to the nuclear translocation of the p50−p65 heterodimer. Thus, these results are consistent with nitrosothiol formation within the p50−p65 heterodimer as the primary mechanism for the inhibition of NF-κB by NO in the intact cell.

Inhibition of p50 Binding to DNA by S-Nitrosylation. S-Nitrosylation of p50 in vitro has previously been shown to inhibit binding to its target κB DNA sites (*8*). To determine whether p50 is S-nitrosylated in vitro under conditions in which NF-κB was inhibited in our cellular studies, p50 was treated with varying concentrations of CSNO (in DTT-free buffer). A dose-dependent decrease in p50 binding to the NF-κB oligonucleotide probe was seen with CSNO treatment (Figure 6). Moreover, the effect of CSNO on p50 DNA binding was reversed if DTT (5 mM) was added after CSNO treatment. These results indicate that SNO inhibits p50 DNA binding by a redox-related modification of a thiol in p50, and they are best rationalized by the formation of a nitrosothiol within the p50 subunit. These data do not, however, definitively exclude alternative redox-related modifications of p50 (*23*).

To prove that p50 was in fact S-nitrosylated and to quantify SNO bound to protein, we assayed directly both the thiol and SNO content of the p50 protein. We determined the number of free thiols in dialyzed recombinant p50 after treatment with sodium cyanoborohydride to be 0.59 ± 0.10 free thiol/p50 molecule by radiolabeling—results consistent with the presence a single free thiol in the DNA binding region of the p50 molecule as previously reported (*6*). Photolysis−chemilumescence analysis showed there to be 1.34 ± 0.26 nitrosothiols/p50 molecule after treatment with CSNO. Thus, our results are consistent with the previous report that a single cysteine residue in the p50 molecule is S-nitrosylated in vitro (*8*), and indicate that this modification is responsible for the decrease in DNA binding seen in our experiments.

Isolation of S-Nitrosylated p50 from Cells under Nitrosative Conditions. To determine if S-nitrosylated p50 could

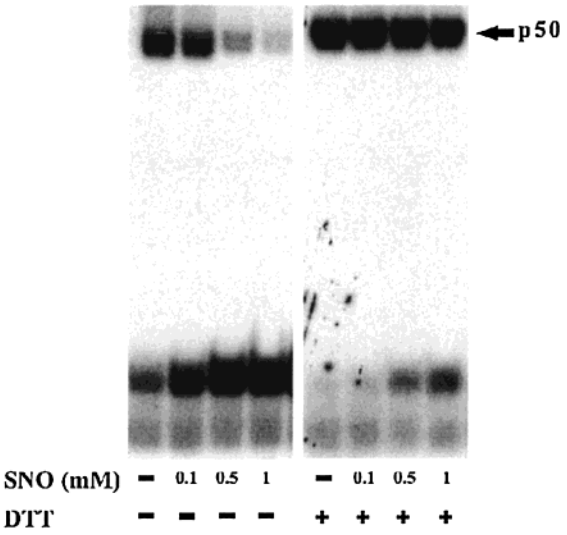

FIGURE 6: SNO inhibits p50 binding to DNA in a thiol-reversible manner. Recombinant p50 protein (1 pmol) was treated with the indicated concentrations of CSNO at 25 °C for 10 min followed by the addition of the NF-κB oligonucleotide and EMSA. When indicated, DTT (5 mM) was added to the reaction mixture after the treatment of p50 with SNO.

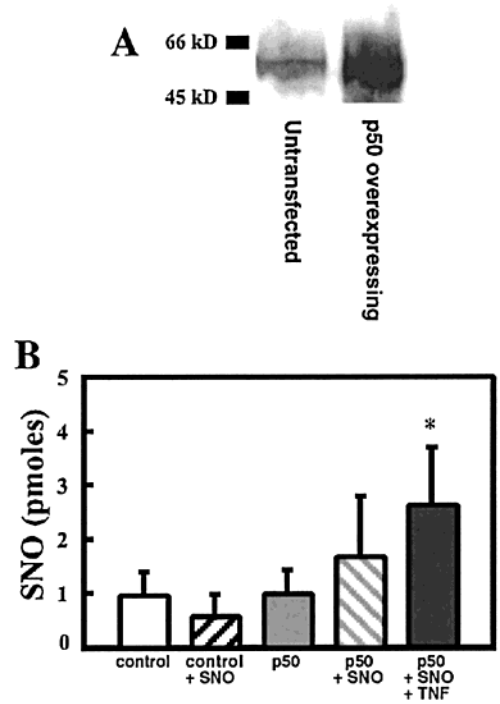

FIGURE 7: Overexpression of p50 increases the nitrosothiol content in p50 IPs. 293 cells were transiently transfected with the p50CMV plasmid using SuperFect. 48 h after transfection, cells were treated with CSNO (0.2 mM) or TNFα (20 ng/mL) + CSNO. Whole cell lysates were then prepared followed by immunoprecipitation of p50 using a rabbit antibody and protein G−Sepharose. (A) p50 Western blot of the whole cell lysates from untransfected and p50CMV-transfected (p50-overexpressing) cells. (B) Nitrosothiol content of the IP eluates from untransfected controls or p50-overexpressing cells after treatment of the cells with SNO or TNFα+SNO. Nitrosothiol content was determined by photolysis−chemiluminescence. ($n = 5$; error bars represent SE.) (*$p < 0.05$ compared to control + SNO.)

be isolated from cells, we immunoprecipitated p50 and measured the nitrosothiol content of the IPs by photolysis−chemiluminescence. S-Nitrosylated proteins were found in the p50 IPs from resting A549 cells, but silver stains showed the presence of non-p50 protein, thus limiting interpretation of the result (data not shown). That is, the data establish the presence of constitutively nitrosylated proteins, but not their identity.

Therefore, to verify that S-nitrosylated p50 is formed in situ, we combined immunoprecipitation with transient overexpression of p50 in 293 cells. Cells overexpressing p50 and untransfected controls were treated with CSNO (500 μM) for 30 min with or without concurrent TNFα-stimulation, and IPs of p50 were then prepared from cell lysates. Immunoblot analysis of the lysates confirmed p50 overexpression (Figure 7A). Low levels of S-nitrosylated protein were found in the IPs from resting 293 cells and from cells overexpressing p50. Interestingly, treatment of cells with CSNO did not significantly increase the constitutive NO content of the IPs from either native or p50-overexpressing cells. More specifically, the p50-overexpressing cells had nearly a 3-fold increase in nitrosothiol content relative to untransfected controls after SNO treatment, but the difference did not reach statistical significance (Figure 7B). In p50-overexpressing cells treated for 30 min with TNFα (20 ng/mL) and CSNO (200 μM), however, the SNO content of the p50 IPs was increased more than 4-fold over IPs from untransfected controls treated with CSNO ($P < 0.05$). These results as a whole indicate that p50 is S-nitrosylated in situ. In addition, our data suggest that concurrent TNFα stimulation increases S-nitrosylation of intracellular p50, perhaps by allowing for greater access of p50 to SNO through degradation of IκBα.

DISCUSSION

Although it is clear that NO bioactivity can regulate NF-κB in situ, the cellular sites and molecular mechanisms of regulation have not been delineated. Previous in vitro studies have shown that S-nitrosylation of the p50 subunit can inhibit DNA binding of NF-κB (p50−p65) (*8, 9*). However, the physiological relevance of p50 S-nitrosylation has not been established. The results presented here suggest that S-nitrosylation is an important regulator of cellular NF-κB function, and provide the first direct evidence that eukaryotic transcription is a site of redox control.

Several converging lines of evidence support this conclusion. First, we found that SNO inhibition of NF-κB DNA binding in cells is reversed by DTT, a compound that is known to remove NO groups from cysteine residues in proteins (*19*−*21*). Such reversibility indicates that a nitrosative or oxidative modification of a cysteine residue in the NF-κB-activating pathway is created by SNO (*23*). Reversal of the SNO-induced inhibitory modification of NF-κB by addition of DTT to the protein extracts points to the p50−p65 heterodimer as the site of modification. That is, if a more proximal protein in the pathway was the target of nitrosylation, then the addition of DTT to extracts would not have restored NF-κB DNA binding. Second, NF-κB (i.e., p50) is nitrosylated by SNO in vitro under the same conditions. Third, although other studies have suggested that NO inhibits NF-κB through increased transcription and stabilization of IκBα (*14, 16*), we saw no effects of SNO on TNFα-induced IκBα degradation. It appears, then, that SNO inhibits NF-κB further downstream in the activation pathway (at least in these cells). That SNO had no effect on the TNFα-induced

nuclear translocation of p65, indicates that the inhibition of NF-κB by SNO occurs at the nuclear level. Last, the isolation of S-nitrosylated p50 from cellular lysates demonstrates directly that this posttranslational modification of the protein indeed occurs in situ.

What is the physiological reason for the NO inhibition of NF-κB? Modification of NF-κB by NO may serve as a negative feedback mechanism that regulates NOS2 transcription. κB sites are present in the NOS2 promoter, and NF-κB binding to these sites is essential for transcription of the NOS2 gene (*11*, *12*). Thus, a negative feedback loop may exist, which couples increased NO production to decreased transcription of the NOS2 gene. Consistent with this hypothesis, NOS inhibition increased NF-κB DNA binding in our studies. NO regulation of NF-κB may also be involved in the control of apoptosis. In particular, the high output NOS2, which is induced by cytokines such as TNFα (*24*), appears, in some cases, to be proapoptotic. Although it is not known how NO activates death programs, there is intriguing evidence for interplay between NO and the TNF receptor family. We previously showed that Fas-induced apoptosis is coupled to denitrosylation of caspase-3 (*25*). We now expand the paradigm by showing that TNFα facilitates S-nitrosylation of NF-κB, perhaps by dissociating it from IκBα, thereby exposing p50 and inhibiting its activation. Since inhibition of NF-κB is known to induce apoptosis (*26*−*28*), NO in this scenario can be viewed as a specific death-effector signal (as opposed to a caustic chemical). These data may explain the observation that increased protein S-nitrosylation is causally linked to cell death in cytokine-activated macrophages (*29*).

Our results indicate that NF-κB can be added to a growing list of proteins whose cellular activity is modified by S-nitrosylation (*20*, *25*, *30*). Of these proteins, NF-κB is most analogous to the bacterial transcription factor OxyR. Oxidation or nitrosylation of a single cysteine at an allosteric site can activate OxyR, resulting in the transcription of antioxidant/antinitrosant genes (*10*, *19*, *31*). NF-κB activation is also regulated by nitrosylation of a single, necessary and sufficient cysteine residue (cysteine 62 on the p50 subunit) (*6*). By contrast with OxyR, however, the target cysteine in p50, cysteine 62, must be in the reduced state for NF-κB to bind to DNA; that is, the modification has inhibitory ramifications. This reduced state is maintained by the ubiquitous intracellular protein thioredoxin in conjunction with the nuclear protein Ref-1 (*32*, *33*).

Our studies with recombinant p50 in vitro confirm that there is one free thiol, that this thiol can be S-nitrosylated in vitro and in vivo, and that this modification may have regulatory implications. The importance of these observations can be understood by appreciating that this is the first time a redox-related modification of a transcription factor has been demonstrated in a eukaryotic cell. More generally, OxyR and NF-κB are the only examples of transcription factors shown to be so modified in vivo. Inasmuch as OxyR is also activated by an (as yet unknown) oxidative modification in vivo, it is possible that alternative redox-related modifications of Cys 62 may modulate NF-κB cellular activity (*23*).

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