**NOS2 Regulation of NF-κB by S-Nitrosylation of p65**

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Signal transduction in the NF-κB transcription factor pathway is inhibited by inducible nitric oxide synthase (NOS2) activity, although the molecular mechanism(s) are incompletely understood. We have previously shown that nitric oxide (NO), derived from NOS2 consequent upon cytokine stimulation, attenuates NF-κB p50-p65 heterodimer DNA binding and have identified the p50 monomer as a locus for inhibitory S-nitrosylation. We now show that the binding partner of p50, NF-κB p65, is also targeted by NO following cytokine stimulation of respiratory epithelial cells and macrophages and identify a conserved cysteine within the Rel homology domain that is the site for S-nitrosylation. S-Nitrosylation of p65 inhibits NF-κB-dependent gene transcription, and nuclear levels of S-nitrosylated p65 correlate with decreased DNA binding of the p50-p65 heterodimer. NOS2 regulates cytokine-induced S-nitrosylation of p65, resulting in decreased NF-κB binding to the NOS2 promoter, thereby inhibiting further NOS2 expression. Collectively, these findings delineate a mechanism by which NOS2 modulates NF-κB activity and regulates gene expression in inflammation.

The transcription factor NF-κB controls the expression of many genes involved in the inflammatory response (1). One of these genes is the inducible nitric oxide synthase (NOS2) whose activity impacts the cellular response to acute injury (2). The product of NOS2, nitric oxide (NO), is known to modulate many genes involved in the inflammatory response (1). One of these proteins includes 1κB kinase β and p50 regulated by this post-translational modification (4, 5). Particularly, we have shown that p50 is S-nitrosylated under conditions of nitrosative stress and is associated with a decrease in NF-κB (p50-p65) DNA binding (4). However, the physiological significance of S-nitrosylation of the NF-κB p50-p65 heterodimer in the context of cytokine signaling and cellular NOS2 expression has not been established.

NOS2 expression is dependent upon NF-κB activation, with the cytokine-responsive κB-binding site(s) identified in both the human and the murine NOS2 promoters (6, 7). Cytokine-stimulated NOS2 activity, in turn, inhibits NF-κB-dependent transcription, but the specific molecular target(s) of NOS2 in the NF-κB pathway have not been elucidated (8). We have previously demonstrated that cytokine-induced NOS activity inhibits NF-κB DNA binding in a reversible manner, a mechanism consistent with S-nitrosylation of the p50-p65 heterodimer (4). Moreover, evidence accumulated recently suggests a central role of S-nitrosylation by NOS2 in the regulation of inflammatory mediators (9, 10).

In the past, the p50 monomer was felt to be the probable target for NOS2-mediated S-nitrosylation of the p50-p65 heterodimer. This rationale was based on the initial identification of a single redox-sensitive cysteine (Cys-62) located in the DNA-binding region of p50 (11). Interestingly, this cysteine is not only conserved throughout all Rel family members but is also found within a canonical SNO motif (12), suggesting the possibility of a common NO-responsive site that could function to universally regulate NF-κB Rel protein-DNA binding (Fig. 1). We now show that the binding partner of p50, p65 (or RelA), is S-nitrosylated in cytokine-stimulated respiratory epithelium and macrophages and identify this conserved cysteine (Cys-38) within the DNA-binding site of the Rel homology domain (RHD) as the site of S-nitrosylation. S-Nitrosylation of p65 is dependent upon NOS2 activity, and nuclear SNO-p65 levels are inversely correlated with NF-κB p50-p65 DNA binding and NF-κB-dependent transcription. These results suggest a pathway by which NOS2 might coordinate the inflammatory response by regulating the NF-κB-dependent transcription of critical response mediators.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Plasmids**—HEK 293 (CRL-1573), RAW 264.7 (TIB-71), and A549 (CCL-185) cells were grown in their ATCC-designated media supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cultures were maintained in 95% air, 5% CO₂ at 37 °C.

Peritoneal macrophages were harvested from 8–12-week-old male C57BL/6 (wild type (WT)) or NOS2 knockout (KO) (B6.129P2-Nos2<sup>m1.Lau</sup>; Jackson Laboratory, Bar Harbor, ME) mice 72 h after intraperitoneal instillation of thioglycollate broth. The peritoneum was lavaged with ice-cold sterile phosphate-buffered saline, cells were collected by centrifugation at 500 × g, and the cell pellet was resuspended in RPMI 1640...
media supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Approximately 1 x 10^7 cells were plated for use in the biotin switch assay.

The p65 wild-type (p65wt) expression plasmid was constructed by inserting full-length human p65 cDNA (a gift from Dr. Albert Baldwin, University of North Carolina) into pDNA-CMV (Clontech) using a BamH1 restriction site. The plasmid expressing p65 with a cysteine → serine mutation at amino acid position 38 (p65C38S) was created utilizing a site-directed mutagenesis kit (QuikChange, Stratagene, La Jolla, CA). The pNFκB-Luc and pRL-CMV (Renilla luciferase) plasmids were purchased from Stratagene and Promega (Madison, WI), respectively.

Cell Lysates—Preparation of A549 and RAW 264.7 cytoplasmic and nuclear extracts was done as outlined previously (13). Whole cell lysates of HEK 293 cells were prepared by resuspending the harvested cells in 1 volume of cold lysis buffer (50 mM HEPES pH 7.9, 150 mM NaCl, 1% Nonidet P-40, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride), placing on ice for 20 min, and pelleting debris by centrifugation at 14,000 x g for 20 min. The protein concentration of the extracts was determined using the BCA method (Pierce Biotechnology), and extracts were used immediately or stored at −80 °C.

NF-κB Reporter Assay—HEK 293 cells were grown to ~75% confluence in 6-well plates. Cells were transfected with 1 μg of pNFκB-Luc and 0.5 μg of pRL-CMV ± 5 μg of p65wt or p65C38S expression plasmids using Lipofectamine 2000 (Invitrogen). Cells were grown for 24 h after transfection prior to the indicated treatments. Luciferase activity was quantitated on a TD 20/20 luminometer using a Dual-Luciferase kit (Promega).

NF-κB DNA Binding Assays—Nuclear protein binding to a consensus NF-κB oligonucleotide was determined using an enzyme-linked immunosorbent assay-based kit (TransAm p65, Active Motif, Carlsbad, CA). Absorbance was read at 450 nm with samples appropriately blanked.

A commercially available kit was utilized for the NF-κB chromatin immunoprecipitation (ChIP) assay (Upstate Biotechnology, Charlotteville, VA). A rabbit antibody directed against NF-κB p65 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunoprecipitation. DNA input was quantified using a 10-fold dilution of the cell lysate. DNA was purified from the NF-κB ChIP eluates as well as input lysate using phenol-chloroform extraction followed by ethanol precipitation. The purified DNA was resuspended in Tris-EDTA and subjected to PCR using primers flanking the cytokine-responsive κB site in the human NOS2 promoter (forward primer, 5′-GGGCTTAT-GTGCCCTAAACCA-3, and reverse primer, 5′-CCACCGG-ACTTGAAGTAAG-3) and mouse NOS2 promoter (forward primer, 5′-ACACGGGCTGAGCTGACTT-3, and reverse primer, 5′-CATTCCACATGGCATGG-3′). PCR products were separated on a 2% agarose gel and visualized by ethidium bromide.

Reverse Transcription-PCR—Total RNA was extracted from cells using the RNeasy Kit (Qiagen, Valencia, CA). Residual genomic DNA was removed by treatment with RNase-free DNase I (Invitrogen). RNA was reverse-transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR amplification of the cDNA was performed using NOS2 (forward, 5′-ACCTTTGT-CACGTACGCCTT-3′; reverse, 5′-CATTTCCCAAATGTTGC- TTGTCT-3′) or β-actin (forward, 5′-TCAAGATCATTGCTC- CTCCTG-3′; reverse, 5′-CTGCTTGCTGATCCACATCG-3′) primers and SYBR Green PCR mix (iQ SYBR Green supermix, Bio-Rad Laboratories). Samples were amplified using a PCR thermocycler (iCycler, Bio-Rad), and the single color real-time PCR detection system (MyIQ, Bio-Rad). Using β-actin as a reference gene, changes in NOS2 mRNA levels between samples were determined.

S-Nitrosodithiol Detection Assay—A biotin switch assay for S-nitrosodithiol detection was performed as described previously (14) with minor modifications. All steps were done with minimal light exposure. 100–250 μg of protein lysate was diluted in HEN buffer (250 mM HEPES-NaOH, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% SDS) with free thiols blocked by the addition of 3 mM methyl methane thiosulfonate followed by heating to 50 °C for 30 min. S-Nitrosothiols were labeled by the addition of 5 mM ascorbic acid and N-(6-(Biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide (EZ-Link Biotin-HDPP, Pierce Biotechnology). Negative controls were prepared by the omission of ascorbic acid. Biotinylated proteins were isolated by incubating overnight (4 °C) with NeutrAvidin agarose beads (Pierce Biotechnology). After extensive washing, proteins were eluted from the beads by heating to 95 °C in Laemmli buffer. Proteins recovered by the biotin switch assay and the input protein lysate were separated by SDS-PAGE and transferred to nitrocellulose, and blots were probed with a rabbit polyclonal antibody to NF-κB p65 (C-20). Immunoreactivity was visualized by enhanced chemiluminescence.

Detection of S-nitrosothiols in p65 immunoprecipitates (IP) by mercury-coupled, photolysis chemiluminescence was performed using a previously described method with minor variations (4). 500 μg of protein lysate was incubated with an antibody to NF-κB p65 (C-20) for 4 h at 4 °C followed by extraction of the immunocomplexes using a commercial kit (Seize IP, Pierce Biotechnology). The IP eluates were kept on ice with limited light exposure prior to analysis of S-nitrosothiols by photolysis chemiluminescence.

S-Nitrosylation of NF-κB p65 by NOS2

| RHD consensus sequence | -5 -3 -1 +1 | PKQRGMRFRYKC EGRSAGSIP |
|------------------------|------------|-------------------------|
| p50/p105               | PKQRGMRFRYVC EGPShGGLP |
| p65                    | PKQRGMRFRYKC EGRSAGSIP |
| c-Rel                  | PKQRGMRFRYKC EGRSAGSIP |
| RelB                   | PKQRGMPFRYEC EGRSAGSIL |
| p52/p100               | PKQRGFRFYRC EGPShGGLP |

FIGURE 1. NO-responsive cysteine in NF-κB RHD. Shown is the amino acid alignment of the NF-κB Rel proteins noting the position of the conserved cysteine (red highlight) in the DNA-binding region of the RHD. Acidic residues at the +1 and acid/base in the −5 to −1 positions (red underline) constitute a motif that favors S-nitrosylation (40, 41). The aromatic amino acid (tyrosine) in position −2 may also facilitate S-nitrosylation formation (12).
**RESULTS**

*S-Nitrosylation of NF-κB p65*—To ascertain whether the NF-κB p65 protein can be modified by S-nitrosylation in situ, we treated untransfected and p65wt-overexpressing HEK 293 cells with S-nitrosocysteine (SNOC, 500 μM) for 1 h followed by quantification of S-nitrosylated p65 (SNO-p65) levels in whole cell lysates using the biotin switch assay. SNO-p65 was detected by immunoblot in untransfected 293 cells only after SNOC treatment (Fig. 2A). On the other hand, HEK 293 cells overexpressing p65wt display some biotin labeling at baseline, indicating the possibility of constitutive S-nitrosylation, but a marked increase in SNO-p65 formation was seen after SNOC treatment. In the absence of concurrent ascorbate treatment, minimal biotin labeling of p65 was noted in the SNOC-treated, p65wt-overexpressing cells, demonstrating the specificity of the biotin switch method for quantifying SNO-p65.

Further corroboration that NF-κB p65 undergoes cellular S-nitrosylation was obtained by mercury-coupled, photolysis chemiluminescence quantification of SNO in p65 IP prepared from SNOC-treated untransfected and p65wt-overexpressing HEK 293 cells. SNO was detected in the p65 IP prepared from untransfected HEK 293 cells only after SNOC treatment (Fig. 2B). In the p65wt-overexpressing cells, SNO levels in the p65 IP increased ~3-fold (33.3 versus 12.2 nM) after SNOC treatment, further substantiating that NF-κB p65 is a target for S-nitrosylation.

**S-Nitrosylation of NF-κB p65 Is Dependent on Cysteine 38**—Previous work has established that the NF-κB p50 monomer undergoes S-nitrosylation at cysteine 62 in the DNA-binding region of the RHD (15). As this cysteine is conserved in the p65 monomer (Fig. 1), we investigated whether this thiol is the target for S-nitrosylation in p65. Using site-directed mutagenesis, we created a p65 expression plasmid encoding a p65 mutant protein that substitutes serine for Cys-38 (p65C38ΔS). We then quantified SNO-p65 levels by biotin switch in untransfected, p65wt-overexpressing, and p65C38ΔS-overexpressing HEK 293 cells with or without SNOC treatment (500 μM). Although a detectable increase in SNO-p65 levels was seen in the untransfected and p65wt-overexpressing cells after SNOC treatment, no change in biotin labeling was observed in the p65C38ΔS-overexpressing cells after SNOC treatment, no change in biotin labeling was observed in the p65C38ΔS-overexpressing cells after SNOC treatment. These results indicate that cysteine 38 is the site of S-nitrosylation in the NF-κB p65 protein (Fig. 3A).

In addition to the RHD DNA-binding site, NF-κB p65 also contains a transactivation domain that functions to initiate NF-κB-dependent transcription when p65-containing dimers are bound to target DNA sites (16). To determine whether S-nitrosylation of p65 results in a decrease in NF-κB-dependent gene transcription, we performed an NF-κB reporter assay in HEK 293 cells overexpressing either the p65wt or the p65C38ΔS protein. Although both p65wt and p65C38ΔS...
expression result in an increase in NF-κB-dependent reporter activity above control, SNOC treatment (500 μM) led to a decrease in NF-κB reporter activity only in the p65wt-expressing cells (relative luciferase activity = 38.4 (−SNO) versus 48.9 (+SNO), p < 0.05) (Fig. 3B). These results are consistent with the prior observation that a serine substitution at the conserved cysteine in the RHD allows for Rel protein DNA binding but a loss of NO-induced binding inhibition (16). Thus, S-nitrosylation at Cys-38 in the p65 protein appears to be a molecular transducer for NO inhibition of NF-κB-dependent gene transcription.

S-Nitrosylation of NF-κB p65 in Cytokine-stimulated Cells—We have previously demonstrated dithiothreitol-reversible, NO-induced inhibition of NF-κB (p50-p65) DNA binding in cytokine-stimulated A549 and RAW 264.7 cells, consistent with S-nitrosylation of the p50-p65 heterodimer (4, 13). Using the biotin switch assay, we now confirm the presence of S-nitrosylated NF-κB p65 in both the cytoplasm and, more importantly, the nucleus of cytokine-stimulated A549 and RAW 264.7 cells. In unstimulated cells, p65 is primarily sequestered in the cytoplasm (presumably bound to IκBα) with a modest degree of basal S-nitrosylation observed in the A549 cells (Fig. 4A). Upon cytokine stimulation, NF-κB p65 undergoes nuclear translocation associated with an increase in NF-κB p65 DNA binding (Fig. 4B). However, at 8 h after stimulation, both the cytoplasmic and the nuclear p65 pools show increased S-nitrosylation in conjunction with the induction of cellular NOS2 expression (Fig. 4A). The increase in nuclear SNO-p65 at 8 h after stimulation coincides with a decrease in NF-κB DNA binding (when compared with 1 h after stimulation) despite there being no change in the total amount of nuclear p65, indicating that cytoplasmic activation of NF-κB is not being affected (Fig. 4B). Thus, S-nitrosylation of NF-κB p65 within the nucleus appears to be a mechanism by which NF-κB DNA binding is regulated in cytokine-stimulated cells.

NOS2 Activity Regulates S-Nitrosylation of p65 and NF-κB DNA Binding—Our prior investigations have shown that NOS2 activity inhibits NF-κB signaling in cytokine-stimulated cells by a mechanism consistent with S-nitrosylation of p50-p65 (4). These observations, along with the fact that cytokine-induced NOS2 expression in A549 and RAW 264.7 cells coincides with a rise in nuclear SNO-p65 levels (Fig. 4A), led us to investigate whether SNO-p65 formation in these cells is NOS2-dependent. Cells were stimulated with cytokines with or without the addition of the NOS2-specific inhibitor 1400W (100 μM). A marked decrease in nuclear SNO-p65 was seen in both cell lines cotreated with 1400W at 8 h after stimulation, indicating that NOS2 activity mediates SNO-p65 formation in cytokine-stimulated cells (Fig. 5A).

To determine whether NOS2 inhibition in cytokine-stimulated cells leads to a decrease in NF-κB binding to target pro-
moter sites, we utilized an NF-κB (p65) ChIP assay. We demonstrate that cytokine stimulation of A549 and RAW 264.7 cells results in an increase in NF-κB binding to the cytokine-responsive NF-κB sites in the NOS2 promoter (Fig. 5B). If the cytokine-stimulated cells are treated concurrently with 1400W (100 μM), NF-κB DNA binding to these κB promoter sites is further augmented. Moreover, the increase in NF-κB binding to the NOS2 promoter elicited by NOS2 inhibition translates into higher cellular NOS2 mRNA transcription and protein expression (Fig. 5, C and D).

To further establish that NOS2 regulates SNO-p65 formation, we quantified SNO-p65 levels in cell lysates prepared from peritoneal macrophages that were harvested from WT (C57BL/6) or NOS2 KO mice. SNO-p65 levels were markedly lower in the NOS2 KO peritoneal macrophages when compared with WT macrophages, both at baseline and after cytokine stimulation, despite an increase in total p65 expression in the NOS2 KO macrophages (Fig. 5E). The decrease in SNO-p65 formation seen in the NOS2 KO macrophages also correlated with an increase in NF-κB DNA binding (Fig. 5F). Collectively, these data indicate that NOS2 functions to control S-nitrosylation of NF-κB p50-p65 in the cell.

**DISCUSSION**

The present study identifies the NF-κB p65 monomer as a target of S-nitrosylation by NOS2 and links the nuclear accumulation of SNO-p65 with inhibition of NF-κB. NF-κB, thus, joins a growing list of immunomodulators that are regulated by NOS2 through S-nitrosylation. In particular, cyclooxygenase-2 (COX-2) undergoes S-nitrosylation via direct interaction with NOS2 in cytokine-stimulated macrophages (9). NOS2 thereby increases COX-2 activity and augments prostaglandin production. In contrast, cytokine-induced, NOS2-mediated S-nitrosylation inhibits the activity of c-Jun N-terminal kinase 1 (JNK1) and the serine kinase Akt, altering the cellular stress response (10, 17). Interestingly, COX-2, JNK1, and Akt have also been shown to influence NF-κB signaling (18–20).

It is increasingly apparent that regulation of inflammation is a primary function for NOS2. Given that NF-κB controls the expression of numerous cytokines, chemokines, adhesion molecules, cell surface receptors, and apoptotic mediators (1), the inhibitory effect of NOS2-mediated S-nitrosylation of NF-κB p65 would be expected to attenuate inflammation. Indeed, selective inhibition of NOS2 has been shown to enhance tissue inflammation in response to acute injury in the lung and other organ systems (21–24). Similarly, mice with genetic deletion of NOS2 have also demonstrated a more pronounced inflammatory response (24–27). In addition to ameliorating inflammation, inhibitory S-nitrosylation of NF-κB p65 would also serve to prevent nitrosative injury by curtailing continued κB-dependent NOS2 expression and cellular NO production. In fact, NOS2 expression has been shown to be augmented in cytokine-stimulated macrophages treated with NOS inhibitors (28), a finding we confirm in our present studies (Fig. 5D), thus linking S-nitrosylation of p65 to the regulation of NOS2 expression.

The fact that two of the five Rel proteins (p65 and p50) have now been shown to be S-nitrosylated at a cysteine conserved in all NF-κB Rel family members implies a general mechanism by which NO might regulate Rel protein-DNA interactions. Prior studies have shown that this cysteine within the RHD must be reduced in order for the NF-κB heterodimer (p50-p65) to bind to specific κB promoter sequences (11). NOS2-mediated S-nitrosylation of this cysteine could function to inhibit Rel protein DNA binding, thereby affecting the transcription of a broad range of inflammatory mediators. Importantly, all of the Rel proteins are also known to modulate NOS2 transcription (7, 29, 30).

NF-κB serves as a prototype of transcription factors whose DNA binding is dependent upon the redox status of protein thiols with several of these transcriptional regulators, including AP-1, Sp1, and cAMP-response element-binding protein, also demonstrating NO-sensitive DNA binding (3). In addition, a growing number of transcription factors have been shown to be modified by S-nitrosylation (31–34). These observations implicate targeted S-nitrosylation as a common mechanism by which gene transcription is controlled. Interestingly, thioredoxin, a protein that governs the redox status of the NF-κB p50-p65 heterodimer, both in the cytoplasm and in the nucleus, is itself regulated by S-nitrosylation (35). In cytokine-stimulated endothelium, S-nitrosylation of thioredoxin has been shown to accelerate thioredoxin activity, which would be expected to promote DNA binding of NF-κB. In this context, SNO-thioredoxin (or perhaps the SNO-metabolizing enzyme, S-nitrosoglutathione reductase (36)) may play a role in the denitrosylation of cytoplasmic NF-κB p65 and 1kB kinase β that is seen upon cytokine stimulation of A549 (Fig. 4A) and Jurkat T cells (5), respectively. However, NOS2 is unlikely to mediate the constitutive S-nitrosylation of NF-κB proteins as it is only expressed in the cell after cytokine activation.

Recent observations implicate dynamic NOS protein-protein interaction in targeting proteins for S-nitrosylation. For example, NOS2 must bind directly to COX-2 in cytokine-stimulated macrophages to induce S-nitrosylation (9). Caspase-3, which is physiologically inhibited by S-nitrosylation, may also bind to NOS2, in this case, in a NO-dependent manner (37). Dexras1 and dynamin are regulated by S-nitrosylation in the context of protein interactions with NOS1 and NOS3, respectively (38, 39). Although we have noted cellular co-localization of NF-κB p65 with NOS2 in both A549 and RAW 264.7 cells (data not shown), direct protein-protein interaction has not been confirmed nor linked with S-nitrosylation of p65. Future investigations will examine this question as well as NOS2 interaction with other NF-κB proteins and transcriptional regulators.

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