Regulation of c-myc gene by nitric oxide via inactivating NF-κB complex in P19 mouse embryonal carcinoma cells

Sung Wook Park and Li-Na Wei*
Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455

Running title: c-myc gene suppression by NO

* Corresponding author:
Li-Na Wei, Dept. Pharmacology, Univ. Minnesota Medical School, 6-120 Jackson Hall 321 Church St. SE, Minneapolis, MN 55455
Tel (612) 6259402 (O), (612) 6258408 (F)
Email “weixx009@tc.umn.edu”
Nitric oxide (NO) may regulate gene expression by directly modifying redox-state sensitive residues of transcription factors. Here we show that the NO donor, sodium nitroprusside (SNP), rapidly represses c-myc gene transcription in a protein-synthesis independent manner in P19 embryonal carcinoma cells, by inactivation of NF-κB. SNP treatment reduces the DNA-binding ability of the constitutively active NF-KB heterodimer, p65/p50, and its consequent transactivation of the c-myc promoter. Repression can be blocked by the peroxynitrite scavenger, deferoxamine (DFO), but not by dithiothreitol (DTT) which triggers reduction of S-nitrosylated residues. In HEK293 cells, where TNF-α can activate NF-κB, SNP likewise suppresses the binding of the active NF-κB complex, restoring the binding of the repressive p50/p50 homodimer complex. This effect of SNP in HEK293 cells is also blocked by DFO. Chromatin immunoprecipitation analysis of SNP-treated P19 cells reveals reduced association of p65, but not of p50, with the promoter region of the endogenous c-myc gene. SNP-induced p65 dissociation was associated with the recruitment of histone deacetylase (HDAC) 1 and 2 to the endogenous c-myc gene promoter and the subsequent deacetylation of its chromatin histone. This study is the first to demonstrate that NO modulates the transcriptional activity of the c-myc gene promoter by dissociating the active form of NF-κB and replacing it with a repressive NF-κB complex, correlated with the recruitment of gene-silencing HDACs. In light of findings that NF-κB stimulates Myc oncoprotein expression in cancers, our findings suggest that NO should be investigated as a prospective therapeutic cancer agent.

Abbreviations: NO, nitric oxide; SNP, sodium nitroprusside; DTT, dithiothreitol; DFO, deferoxamine; HDAC(s), histone deacetylase(s); TNF-α, tumor necrosis factor-α; URE, upstream responsive element; IRE, internal responsive element; ActD, actinomycin D; CHX, cycloheximide; RT-PCR, reverse transcriptase-coupled polymerase chain reaction; ChIP, chromatin immunoprecipitation.
Nitric oxide (NO) is a signaling molecule involved in a wide spectrum of pathophysiological processes such as inflammation, apoptosis, regulation of enzyme activity, and gene expression. NO may modulate the cellular redox state by acting as an oxidant, thereby activating or inhibiting various enzymes involved in a number of signal transduction pathways. It is known that NO can regulate gene expression by modulating transcription factors, the translation or stability of mRNA (1) and the modification of proteins (2-4). It is also known that these effects of NO are differentially elicited by various concentrations of NO in the microenvironment. For instance, a low level of NO activates the cGMP second messenger system, namely the cGMP-dependent protein kinase pathway, in vascular smooth muscle and others (5-7). However, a high level of NO, such as that generated by inducible NO synthase or administered exogenously, will directly modify target proteins through S-nitrosylation of cysteine residues or nitration of tyrosine (Tyr) residues (2-4, 8, 9). We have previously identified a novel inhibitory role for NO in the transcription of the mouse kappa opioid receptor (KOR) gene in P19 embryonal carcinoma cells - a role mediated primarily through reduced expression of c-Myc that activates KOR gene transcription (10).

c-Myc is a transcription factor of the basic-helix-loop-helix-leucine zipper family. It forms a heterodimer with Max on the E-box of target gene promoters to activate transcription. In contrast, a heterodimer of Max and Mad binds to the E-box to suppress transcription. A functional role for c-Myc has been established in a wide variety of cellular processes, including proliferation, metabolism, apoptosis, differentiation, genomic stability (11-14) and cell cycle progression from quiescent to synthetic phases (15, 16). Our recent study of P19 stem cells presented evidence that c-Myc functions as an activator for the mouse KOR gene by binding to its promoter. We also showed that NO suppressed KOR expression by rapidly down-regulating c-Myc protein synthesis in P19 cells (10). This result prompted us to investigate the mechanisms underlying rapid down-regulation of c-Myc by NO in P19 cells.

Regulation of c-myc gene expression has been shown to occur at multiple levels, including gene transcription, premature termination of translation (17, 18), and translocation (19, 20). In Burkitt’s lymphoma the c-myc gene is translocated to the immunoglobulin heavy chain gene loci on either chromosome 2, 14, or 22. Transcription
of the mouse gene can be initiated from two promoters, P1 and P2, separated by approximately 160 bp’s. P2 is the major promoter where several transcription factors and regulatory DNA elements have been identified. For instance, three cis-acting elements for ME1a2, E2F and ME1a1 have been found in P2 (21, 22). The pituitary tumor-transforming gene (PTTG) product can bind to a region near the P2 initiation site as a protein complex containing the upstream stimulatory factor-1 (23). A Smad-responsive element has been shown to be responsible for TGF-β mediated suppression of c-myc transcription (24). An intron I X box is known to be involved in leukemia HL-60 differentiation (25, 26). In addition, two functional NF-κB elements have been identified in both the human and the murine c-myc genes (27-29), one located from –1261 to –1251 in the upstream region of P2 and the other located from +280 to +289. Importantly, transcription of the translocated c-myc gene in Burkitt’s lymphoma is stimulated by constitutively expressed NF-κB (19). Our finding of rapid repression of c-Myc by NO in P19 cells (10) and the identification of two NF-κB binding sites in the c-myc promoter prompted us to examine the potential role of NF-κB in mediating NO regulation of c-myc in P19 cells.

NF-κB is an inducible transcription factor. Five members of NF-κB are known, including p50 (NF-κB1), p52 (NF-κB2), c-Rel, RelB and p65 (RelA). The major active form of this transcription factor is the p65/p50 heterodimer (30-32). In most cells, NF-κB complexes are predominantly cytoplasmic, and are usually associated with an inhibitory protein, the IκB family. Upon stimulation by pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) or interleukin-1, NF-κB dimer dissociates from IκB and is translocated into nuclei to act on the target gene (33, 34). Translocation into nuclei is considered to be a general, underlying mechanism for the activation of NF-κB in gene regulation. It has been shown that NO can inhibit NF-κB by modifying the p50 subunit via redox-based S-nitrosylation of a cysteine residue in its N-terminal region. This effect of NO is sensitive to dithiothreitol (DTT) (4, 35, 36). In our previous study, the effect of NO on c-Myc in P19 cells was not blocked by DTT (10), indicating the involvement of other NO-triggered mechanisms in the regulation of NF-κB activity.
In this study, we found that a NO donor sodium nitroprusside (SNP) repressed the DNA binding and activating function of the constitutively active p65/p50 form of NF-κB in P19 cells. Although not affected by DTT, it was blocked by deferoxamine (DFO), a peroxynitrite scavenger. Similarly, the NO donor also repressed TNF-α-activated NF-κB in cells where NF-κB normally existed as an inactive complex. The effect of NO in these cells was likewise blocked by DFO. We then confirmed the effect of NO on NF-κB and the c-myc promoter by examining the dynamic behavior of the NF-κB complex and histone deacetylases (HDACs), and chromatin acetylation on the endogenous c-myc promoter. This study demonstrates, for the first time, that NO modulates the transcription of the c-myc gene by inducing the dissociation of p65/p50 NF-κB from the c-myc promoter region and the recruitment of the repressive p50 homodimer complex, in correlation with increased recruitment of HDACs and decreased histone acetylation on the endogenous c-myc promoter.
EXPERIMENTAL PROCEDURES

Antibodies and Plasmids Antibodies (Abs) against c-Myc (N-262), p65 (H-286), HDAC1 (C-19), HDAC2 (H-54) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-p50 (06-886) and anti-acetylated histone H4 (06-866) were from Upstate Biotechnology (Lake Placid, NY). The expression vectors, pMT2T-p65 and pMT2T-p50, were kind gifts from Dr. U. Siebenlist (NIH, MD) (37), pcDNA-His-p65 was a kind gift from Dr. A. Carter (University of Iowa, IW) (38) and NF-κB specific reporter construct, p(Igk)4-LUC, that contains 4 tandem repeats of NF-κB binding sites was a gift from Dr. N. Mackman (Scrrips Institute, CA) (39).

Cell Culture and Transient Transfection P19 embryonal carcinoma cell and COS-1 cell lines were maintained as described previously (10). HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetus bovine serum and 1% nonessential amino acids. Transient transfection was performed in P19 and COS-1 cells using calcium chloride method and reporter assays were conducted as described previously (40).

Analyses of c-myc mRNA The endogenous c-myc mRNA was analyzed by a reverse transcriptase-coupled polymerase chain reaction (RT-PCR) as described (10). Total RNA was isolated, by using a Trizol kit (Invitrogen), from P19 cells which were pretreated with 2 μg/ml of actinomycin D (ActD), or 2 μg/ml of cycloheximide (CHX). Five mM DTT or 0.1 mM DFO was added for 30 min prior to the addition of 0.5 mM SNP for 6 h. To determine c-myc mRNA stability, P19 cells were treated with 2 μg/ml of ActD for different durations in the presence of 1 mM SNP for 6 h. Primers specific to c-myc mRNA for the amplification procedure are 5’-CCATATGCCCCTCAACGTGAAC-3’ and 5’-GGGATCCTTATGCACCAGAGTTT-3’, which span a 1350 bp coding region.

Electrophoretic Mobility Shift Assay (EMSA) and Western Blot Nuclear extracts and whole cell lysates were isolated from P19 cells treated as above. HEK293 cells were treated with 10 ng/ml TNF-α for 8 h to activate NF-κB, followed by the addition of SNP for 6 h. EMSA was performed as described (41). Four types of double-stranded
oligonucleotides (URE, IRE, mt-URE, and nonspecific KOR gene promoter sequences) (Fig. 2) were each used as probes that were labeled with $[^{32}\text{P}]d\text{CTP}$, or used as competitors without labeling. Whole cell lysates were analyzed on a Western blot by using anti-c-Myc antibody as described (10).

*Chromatin Immunoprecipitation (ChIP) Assays* P19 cells were treated with SNP or SNP plus DFO for 6 h (or in a time-dependent manner), followed by cross-linking with 1% formaldehyde. ChIP assay was as described previously (10) by using 2 μg of anti-AcH4, anti-p65, anti-p50, anti-HDAC1, or anti-HDAC2 antibodies. Precipitated DNA was amplified with primers specific to the URE flanking sequences in the endogenous c-*myc* promoter and to its 3’ untranslated region (UTR), followed by Southern blot analyses of amplified endogenous c-*myc* sequences.
RESULTS

Transcriptional Repression of c-myc Gene by NO[1] We have previously shown that several NO donors suppressed KOR gene expression in P19 cells through repressing c-Myc protein expression and its binding to the KOR promoter (10). To determine if the repression of c-Myc by NO in P19 cells occurred at transcriptional or post-transcriptional levels, P19 cells were treated with SNP, a NO donor, in the absence or presence of ActD or CHX followed by analyses of c-myc mRNA level with an established RT-PCR (Fig. 1A). NO dramatically reduced the constitutively expressed c-myc mRNA level (lane 2), and ActD effectively blocked c-myc transcription in the absence (lane 3) or presence (lane 4) of the NO donor SNP. Interestingly, CHX, although slightly increased c-myc expression (lane 5), had little effect on the repression elicited by NO (lane 6), indicating that NO directly repressed c-myc transcription and that protein synthesis was probably not involved in this repression. To examine if c-myc mRNA stability was affected by NO, an experiment was carried out to determine c-myc mRNA half-life by treating P19 cells with ActD, in the presence of SNP for various periods of time. As shown in Fig. 1B, the c-myc mRNA half-life remained at approximately 20 min in both control cells (lanes 1-5) and cells exposed to NO (lanes 6-10). Therefore, c-myc mRNA stability is not affected by NO, whereas its gene transcription is directly repressed by NO signal.

Repression of c-myc Gene Transcription by NO through Inactivation of NF-κB Activity[2] To define the target of NO-mediated repression of c-myc gene transcription, the regulatory regions of c-myc gene was carefully examined. This gene utilizes two promoters located approximately 160 bp apart, with the second promoter residing in exon 1. Interestingly, two putative NF-κB binding sites, an upstream responsive element (URE) (GGGTTTCCCCC) located at −1261 to −1251 bp in the 5’-flanking region of promoter 1, and an internal responsive element (IRE) (GGGAATTTTT) located at +280 to +289 relative to the promoter P2 (27, 28), were found to be relevant (Fig. 2A). NF-κB has been shown to be a target protein of NO signal (3, 42). We examined how the endogenous NF-κB activity in P19 cells might be affected by NO and augment c-myc gene expression. P19 cells were treated with SNP, followed by analyzing its nuclear extract for DNA binding ability to the κB binding sites of the c-myc promoter in EMSA. As shown
in Fig. 2B, the $^{32}$P-labeled URE probe revealed a major retarded band and several minor bands (lane 1), which could be competed out specifically by the wild type cold probe (lane 2), but not by the mutated cold probe (mt-URE, lane 3) or nonspecific oligonucleotides (lane 4). The anti-p65 antibody was able to block the major binding species that is the heterodimeric p65/p50 NF-$\kappa$B complex (lane 5), and the anti-p50 antibody was able to supershift the minor species, the p50 complex (lane 6). The control, a non-specific antibody such as the anti-Sp1, had no effect (lane 7). Therefore, the URE consensus sequences of the c-myc gene indeed can be bound by p65/p50 heterodimer and p50 homodimer of NF-$\kappa$B, but the major complex in P19 cells is the transcriptionally active p65/p50 heterodimer. The second putative NF-$\kappa$B binding site, IRE, was also tested in EMSA as shown in Fig. 2C, which appeared to share a very similar binding pattern as that of the URE. Furthermore, SNP decreased the intensity of the p65/p50 NF-$\kappa$B band but increased the p50 complex for both NF-$\kappa$B sites (lanes 1-4), revealing a decreased activator p65/p50-DNA interaction of P19 nuclear extract as a result of SNP treatment.

To determine if the transcriptional activity of endogenous NF-$\kappa$B was affected by NO, transient transfection assays were conducted in P19 stem cells and fully differentiated COS-1 cells by using a specific NF-$\kappa$B reporter, p(Igk)4-LUC (39), without introducing any other expression vectors. As shown in Fig. 3A, SNP effectively suppressed the specific reporter activity in a dose-dependent manner in P19 cells, but not in COS-1 cells, in consistence with our previous conclusion that NO suppressed KOR gene transcription only in P19 cells but not in COS-1 cells (10). This suppressive effect of NO on NF-$\kappa$B reporter plasmid in P19 cells agreed with the reduced DNA binding activity of NF-$\kappa$B from SNP-treated P19 cells (Fig. 2C). The resistance of the same reporter activity to SNP in COS-1 cells was due to the absence of p65/p50 species binding to the URE in COS-1 cells that contained primarily the p50 species binding to the URE site (data not shown). To further confirm that the effect of SNP was indeed mediated by p65/p50 NF-$\kappa$B, the specific reporter and a pMT2T-p65 expression vector (37) were simultaneously introduced into COS-1 cells (Fig. 3A). Indeed, ectopic expression of p65 increased the specific reporter activity in COS-1 cells, which was again
suppressed by SNP in a dose-dependent manner (Fig. 3B). Thus, NO not only suppresses the DNA binding activity of p65/p50 NF-κB, but also efficiently reduces NF-κB activity in transcriptional control. The effect is primarily at the p65 subunit.

*Repression of c-myc Gene Transcription by NO through Peroxynitrite* Previously we have suggested that S-nitrosylation was not involved in c-myc repression by SNP, a NO donor, in P19 cells because this suppressive effect could not be reversed by DTT, an agent that blocks S-nitrosylation of Cys residues by NO (10). Although it is accepted that NF-κB activity can be inhibited by NO through S-nitrosylation on a Cys residue of its p50 subunit (35, 36), the failure of DTT to reverse the repressive effects of SNP in P19 cells raised the question as to what other pathway could be involved in this novel suppressive effect of SNP in P19 cells. The alternative modification elicited by NO is Tyr nitration triggered by the highly reactive peroxynitrite (ONOO⁻) generated from NO and superoxide (4). To test this possibility, we treated P19 cells with DTT, or DFO that is known to scavenge peroxynitrite, followed by examining the expression of c-myc mRNA as shown in Fig. 4. DTT failed to reverse the repressive effect of SNP on c-myc transcription (Fig. 4A, lane 3), whereas DFO efficiently blocked the repressive effect of SNP (lane 4). Consistently, c-Myc protein expression (Fig. 4B) was repressed by SNP (lane 2), which was effectively reversed by DFO (lane 4), but not by DTT (lane 3). The potent cGMP analog 8-bromo-cGMP and guanylate cyclase inhibitor ODQ affected neither c-myc mRNA nor its protein level (data not shown). In order to confirm whether peroxynitrite was indeed responsible for inactivating NF-κB activity, transient transfection assays were performed using the specific reporter, p(Igk)4-LUC, in P19 cells. As shown in Fig. 4C, DTT failed to reverse the repressive effect of SNP on the NF-κB reporter, but DFO very efficiently reversed the repression to approximately 75% of the control level. These results have ruled out the S-nitrosylation and cGMP-dependent pathways, and suggested that peroxynitrite generated from NO could play a role in the repression of c-myc transcription in P19 cells, which may involve nitration of NF-κB.

It was noted that changes had occurred in DNA binding pattern of endogenous NF-κB and p50 homodimer in P19 cells following SNP treatment (Fig. 1C). To further confirm this point, we made use of HEK293 cells where the endogenous NF-κB existed
primarily as the inactive complexes and could be activated by TNF-α. By testing DNA binding patterns of HEK293 extract as shown in Fig. 5, the portion of heterodimeric NF-κB increased in response to TNF-α stimulation as predicted (lane 2), which was blocked by SNP and replaced with the p50/p50 homodimer complex after SNP treatment (lane 3). Furthermore, the SNP-triggered blockage of NF-κB heterodimer formation was again reversed by DFO (lane 4). The identity of p65/p50 heterodimer and p50/p50 homodimer was again confirmed by using specific antibodies (lanes 5-8) which either blocked (for p65/p50 heterodimer) or super-shifted (for p50/p50 homodimer) the complexes. This result confirms that indeed, SNP triggers the dissociation of the p65/p50 complex from, and the association of p50 complex with, the target DNA, in consistence with the effects of SNP and DFO in the regulation of c-myc gene, as well as its reporter, in P19 cells (Fig. 4).

The results of the two cell lines have demonstrated that peroxynitrite indeed disrupts DNA binding of the activated NF-κB, i.e. the p65/p50 heterodimer, which can be blocked by DFO, a scavenger of peroxynitrite.

**Recruitment of HDACs 1 and 2 to the Endogenous c-myc Promoter by SNP Treatment** The activity of transcription factors can be modulated by altering the recruited coregulators, in addition to changes in DNA binding ability. For the repressive activity of p50 homodimer, the recruitment of HDACs has been shown to play a major role (43, 44). To confirm the effect of SNP, and thus replacement of p65/p50 with p50/p50, on its target gene, the role of HDACs activity was examined. P19 cells were transfected with the specific NF-κB reporter p(Igk)4-LUC, and treated with SNP, trichostatin A (TSA) or in combination. As shown in Fig. 6, TSA alone induced this reporter (lanes 4 and 5) and rescued the repressive effect of SNP (lanes 1-3 and 6-8), confirming the involvement of HDACs in the repressive activity of NO on NF-κB target gene's transcription.

To further examine the association pattern of HDACs with the affected promoter in cells treated with SNP, ChIP assay was performed. This experiment was conducted to examine histone acetylation (Fig. 7A) status and association of p65, p50 and HDACs (Fig. 7B) with the endogenous c-myc gene promoter in P19 cells, mediated through NF-κB.
binding site URE. The top panel in Fig. 7A shows the relative primer positions used to amplify the endogenous c-myc sequences surrounding the URE site, as well as its 3'-UTR, which was precipitated in the immune complexes. As shown in Fig. 7A middle panel, treatment of P19 cells with SNP caused a dramatic decrease in the level of histone acetylation on the endogenous c-myc sequences flanking the URE site (lane 3), and addition of DFO completely recovered histone acetylation in this region (lane 4). As a control, histone acetylation was not detected in its 3' UTR (bottom panel). Fig. 7B showed that in control cells, p65 and p50 were associated with the endogenous c-myc promoter regions flanking the URE site (top, left panel), but not the 3'-UTR (top, right panel). In SNP-treated cells, p65, but not p50 association was dramatically reduced. By using anti-HDAC1 and anti-HDAC2 antibodies in ChIP assays, it was apparent that both HDAC1 and HDAC2 were recruited to the endogenous c-myc promoter regions, but HDAC1 appeared to be preferentially recruited (bottom panels). Thus, SNP treatment triggers dissociation of p65, but not p50, from the endogenous c-myc promoter, correlated with increased HDACs recruitment to and decreased histone acetylation of this promoter.

To follow the dynamic behavior of transcription complexes on the endogenous c-myc promoter immediately after exposure to SNP, ChIP assays were conducted to examine p65 and acetylated histones of the endogenous c-myc promoter every 30 minutes following SNP treatment. The dissociation of p65 subunit from, and histone deacetylation of, the endogenous c-myc promoter started between 90 and 120 minutes following SNP treatment (Fig. 7C). Thus, SNP treatment triggers early cofactor exchange on the endogenous c-myc promoter, in particular dissociation of p65 and recruitment of HDACs, resulting in rapid histone deacetylation of this promoter.

DISCUSSION

Eukaryotic cells respond to a variety of extracellular signals and environmental stresses. These signals are coordinated at various levels and finally delivered to transcription factors for a general or cell-type specific response. NF-κB is an inducible transcription factor that has attracted considerable attention because of its role in a wide variety of cellular processes. It is the major player in immune and stress responses, and,
in particular, NO-elicited pathophysiological effects (32, 33). It is generally believed that S-nitrosylation of the p50 subunit is responsible for the effect of NO on NF-κB activity (3, 35, 36, 42). Our study presents the first evidence for a potentially important role for NO-induced modification of p65/p50 NF-κB activity, which is shown here to modulate c-myc gene transcription. We propose that SNP exerts at least two important effects on NF-κB, i.e. reduced DNA binding of the p65/p50 heterodimer and replacement of the p65/p50 with the repressive p50/p50 homodimer, leading to enhanced recruitment of HDACs. Our study also reveals, for the first time, that the proto-oncogene c-myc is an endogenous NF-κB target gene, as demonstrated by ChIP analyses of the endogenous c-myc gene promoter.

The effect of NO to inhibit NF-κB activity and subsequently repress c-myc transcription in P19 cells, but not COS-1 cells, suggests that NF-κB is constitutively active in P19 stem cells but is inactive in differentiated COS-1 cells. Consistent with this assumption, P19 cells show constitutive histone hyperacetylation of the c-myc gene promoter regions surrounding the κB sites (Fig. 7). As such, neither TNF-κ nor lipopolysaccharide could further stimulate NF-κB activity in P19 cells (data not shown). EMSA studies reveal that in P19 stem cells where the c-myc gene is constitutively active, the p65/p50 heterodimer is the major species of NF-κB complex that binds to the κB binding sites on the c-myc promoter (Fig. 2). This observation may be physiologically relevant, as elevated NF-κB activity is detected in numerous solid tumor cell lines and human cancers (45-48). In contrast, the p50 homodimer appears to be the major complex binding to the target promoter in fully differentiated HEK293 cells (44) where NF-κB is normally inactive and where TNF-κ is able to trigger the formation of the active p65/p50 heterodimer (Fig. 5). In COS-1 cells, where most endogenous NF-κB subunits are also of the p50/p50 species, ectopic expression of p65 dramatically elevated NF-κB reporter gene activity, indicating that additional p65 activates NF-κB (Fig. 3). Furthermore, this activation was suppressed by the NO donor SNP. Therefore, SNP exerts a similar repressive effect on both constitutive and TNF-κ-stimulated active p65/p50 NF-κB complex, leading to the dissociation of NF-κB from its target DNA and the replacement with the repressive p50/p50 complex.
NF-κB activity can be modulated by a number of events, including changes in the status of the inhibitory protein IκB, its shuttling between the cytoplasm and the nucleus, and several types of protein modification such as phosphorylation (49-51), acetylation (52, 53), and S-nitrosylation (35, 36). It is generally believed that the primary mode of NF-κB modification by NO is through S-nitrosylation of the p50 subunit and subsequent reduction in its DNA binding ability. However, because SNP exerted no effects on COS-1 cells, where p50/p50 homodimers represent the major species of NF-κB (data not shown), it is tentative to speculate that the p65/p50 heterodimer, not the p50/p50 homodimer, may be the preferred target of SNP-elicited suppression. Moreover, DTT could not reverse SNP repression of c-myc, reporter gene activities, or DNA binding activity of NF-κB (Figs. 4 and 5), but a peroxynitrite scavenger, DFO, was able to do so (Fig. 4). These results rule out S-nitrosylation as the underlying mechanism, and suggest that Tyr nitration could be the actual underlying mechanism for this novel effect of NO. It is proposed that an alternative pathway of protein modification by NO, possibly through Tyr nitration of the p65 subunit of NF-κB, is able to rapidly inactivate NF-κB activity. We are in the process of determining specific Tyr residue(s) of NF-κB that could be NO targets.

NO is known to be involved in numerous pathophysiological processes. In particular, Tyr nitration of various proteins by peroxynitrite has been detected in a number of inflammatory or degenerative diseases (54, 55), and tumors (9, 56). As such, it has generated enthusiasm for managing diseases with agents that modulate NO signaling pathways. NF-κB is an important transcription factor that coordinates the expression of a wide variety of genes (57), and is known to be an inhibitor of apoptosis, because of its ability to induce anti-apoptotic factors such as cellular inhibitors of apoptosis (cIAPs) and the members of the BCL2 family (58, 59). The c-Myc protein is involved in numerous important cellular processes, including proliferation, metabolism, cell cycle control, apoptosis, differentiation, genomic stability and tumor formation (11-13, 60, 61). The ability of a NO donor, SNP, to directly and effectively repress NF-κB activity provides a potentially important route for therapeutic intervention of numerous disease conditions. In particular, its rapid transcriptional repression of c-myc gene via inactivating
NF-κB could provide an efficacious strategy for the use of NO donors in managing tumors.
Acknowledgements

This study was supported by NIH grants DA11190, DA11806, DA13926, DK 54733, and DK60521 to LNW. We thank generous gifts of expression vectors and reporters from Drs. U. Siebenlist, A. Carter, and N. Mackman. We thank Dr. F. Burton for his critical reading of this manuscript.
REFERENCES

1. Bouton, C., and Demple, B. (2000) J. Biol. Chem. 275, 32688-32693
2. Shindler, H., and Bogdan, C. (2001) Int. Immunopharmacol. 1, 1443-1455
3. Marshall, H. E., Merchant, K., and Stamler, J. S. (2000) FASEB J. 14, 1889-1900
4. Bogdan, C. (2001) Trends Cell Biol. 11, 66-75
5. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 9265-9269
6. Gu, M., Lynch, J., and Brecher, P. (2000) J. Biol. Chem. 275, 11389-11396
7. Lincoln, T. M., Dey, N., and Sellak, H. (2001) J. Appl. Physiol. 91, 1421-1430
8. Hausladen, A., Privalle, C. T., Keng, T., DeAngelo, J., and Stamler, J. S. (1996) Cell 86, 719-729
9. Haqqani, A. S., Kelly, J. F., and Birnboim, H. C. (2002) J. Biol. Chem. 277, 3614-3621
10. Park, S. W., Li, J., Loh, H. H., and Wei, L. N. (2002) J. Neurosci. 22, 7941-7947
11. Dang, C. V. (1999) Mol. Cell. Biol. 19, 1-11
12. Eisenman, R. N. (2001) Genes Dev. 15, 2023-2030
13. Pelengaris, S., Littlewood, T., Khan, M., Elia, G., and Evan, G. (1999). Mol. Cell 3, 565-577
14. Pelengaris, S., Khan, M., and Evan, G. (2002) Nat. Rev. Cancer 2, 764-776
15. Eilers, M., Schirm, S., and Bishop, J. (1991) EMBO J. 10, 133-141
16. Mateyak, M. K., Obaya, A. J., and Adachi, S. (1997) Cell Growth Differ. 8, 1039-1048
17. Krumm, A., Meulia, T., Brunvand, M., and Groudine, M. (1992) Genes Dev. 6, 2201-2213
18. Perez-Juste, G., Garcia-Silva, S., and Aranda, A. (2000) J. Biol. Chem. 275, 1307-1314
19. Kanda, K., Hu, H. M., Zhang, L., Grandchamps, J., and Boxer, L. M. (2000) J. Biol. Chem. 275, 32338-32346
20. Ratsch, A., Joos, S., Kioschis, P., and Lichter, P. (2002) Exp. Cell Res. 273, 12-20
21. Moberg, K. H., Tyndall, W. A., and Hall, D. J. (1992) J. Cell. Biochem. 49, 208-215
22. Rhee, K., Ma, T., and Thompson, E. A. (1994) J. Biol. Chem. 269, 17035-17042
23. Pei, L. (2001) J. Biol. Chem. 276, 8484-8491
24. Yagi, K., Furuhashi, M., Aoki, H., Goto, D., Kuwano, H., Sugamura, K., Miyazono, K., and Kato, M. (2002) J. Biol. Chem. 277, 854-861
25. Pan, Q., and Simpson, R. U. (1999) J. Biol. Chem. 274, 8437-8444
26. Chen, L., Smith, L., Johnson, M. R., Wang, K., Diasio, R. B., and Smith, J. B. (2000) J. Biol. Chem. 275, 32227-32233
27. Kessler, D. J., Duyao, M. P., Spicer, D. B., and Sonenshein, G. E. (1992) J. Exp. Med. 176, 787-792
28. La Rosa, F. A., Pierce, J. W., and Sonenshein, G. E. (1994) Mol. Cell. Biol. 14, 1039-1044
29. Ji, L., Arcinas, M., and Boxer, L. M. (1994) Mol. Cell. Biol. 14, 7967-7974
30. Chen, F. E., Huang, D. B., Chen, Y. Q., and Ghosh, G. (1998) Nature 391, 410-413
31. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. (2002) Nat. Rev. Cancer 2, 301-310
32. Li, Q., and Verma, I. M. (2002). Nat. Rev. Immunol. 2, 725-734
33. Ghosh, S., and Karin, M. (2002) Cell 109, S81-S96
34. Chen, G., and Goeddel, D. V. (2002) Science 296, 1634-1635
35. Matthews, J. R., Botting, C. H., Panico, M., Morris, H. R., and Hay, R. T. (1996) Nucleic Acid Res. 24, 2236-2242
36. DelaTorre, A., Schroeder, R. A., Punzalan, C., and Kuo, P. C. (1999) J. Immunol. 162, 4101-4108
37. Franzoso, G., Bours, V., Park, S., Tomita-Yamaguchi, M., Kelly, K., and Siebenlist, U. (1992) Nature 359, 339-342
38. Carter, A. B., Knudtson, K. L., Monick, M. M., and Hunninghake, G. W. (1999) J. Biol. Chem. 274, 30858-30863
39. O'Connell, M. A., Bennett, B. L., Mercurio, F., Manning, A. M., and Mackman, N. (1998) J. Biol. Chem. 273, 30410-30414
40. Hu, X., Bi, J., Loh, H. H., and Wei, L. N. (2001) *J. Biol. Chem.* **276**, 4597-4603
41. Li, J., Park, S. W., Loh, H. H., and Wei, L. N. (2002) *J. Biol. Chem.* **277**, 39967-39972
42. Colasanti, M., and Persichini, T. (2000) *Brain Res. Bull.* **52**, 155-161
43. Ashburner, B. P., Westerheide, S. D., and Baldwin, A. S. Jr. (2001) *Mol. Cell. Biol.* **21**, 7065-7077
44. Zhong, H., May, M. J., Jimi, E., and Ghosh, S. (2002) *Mol. Cell* **9**, 625-636
45. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J. Jr., and Sledge, G. W. Jr. (1997). *Mol. Cell. Biol.* **17**, 3629-3639
46. Kim, D. W., Sovak, M. A., Zanieski, G., Nonet, G., Romieu-Mourez, R., Lau, A. W., Hafer, L. J., Yaswen, P., Stampfer, M., Rogers, A. E., Russo, J., and Sonenshein, G. E. (2000) *Carcinogenesis* **21**, 871-879
47. Cogswell, P. C., Guttridge, D. C., Funkhouser, W. K., and Baldwin, A. S. Jr. (2000) *Oncogene* **19**, 1123-1131
48. Baldwin, A. S. (2001) *J. Clin. Invest.* **107**, 241-246
49. Wang, D., Westerheide, S. D., Hanson, J. L., and Baldwin, A. S. Jr. (2000) *J. Biol. Chem.* **275**, 32592-32597
50. Jang, M. K., Goo, Y. H., Sohn, Y. C., Kim, Y. S., Lee, S. K., Kang, H., Cheong, J., and Lee, J. W. (2001) *J. Biol. Chem.* **276**, 20005-20010
51. Zhong, H., Voll, R. E., and Ghosh, S. (1998) *Mol. Cell* **1**, 661-671
52. Chen, L. F., Fischle, W., Verdin, E., and Greene, W. C. (2001) *Science* **293**, 1653-1657
53. Furia, B., Deng, L., Wu, K., Baylor, S., Kehn, K., Li, H., Donnelly, R., Coleman, T., and Kashanchi, F. (2002) *J. Biol. Chem.* **277**, 4973-4980
54. Estevez, A. G., Crow, J. P., Sampson, J. B., Reiter, C., Zhuang, Y., Richardson, G. J., Tarpey, M. M., Barbeito, L., and Beckman, J. S. (1999) *Science* **286**, 2498-2500
55. Reiter, C. D., Teng, R. J., and Beckman, J. S. (2000) *J. Biol. Chem.* **275**, 32460-32466
56. Yamamoto, T., Maruyama, W., Kato, Y., Yi, H., Shamoto-Nagai, M., Tanaka, M., Sato, Y., and Naoi, M. (2002) *J. Neural. Transm.* **109**, 1-13
57. Li, Q., and Verma, I. M. (2002) Nat. Rev. Immunol. 2, 725-734
58. Grossmann, M., O'Reilly, L. A., Gugasyan, R., Strasser, A., Adams, J. M., and Gerondakis, S. (2000) EMBO J. 19, 6351-6360
59. Karin, M., and Lin, A. (2002) Nat. Immunol. 3, 221-227
60. D'Cruz, C. M., Gunther, E. J., Boxer, R. B., Hartman, J. L., Sintasath, L., Moody, S. E., Cox, J. D., Ha, S. I., Belka, G. K., Golant, A., Cardiff, R. D., and Chodosh, L. A. (2001) Nat. Med. 7, 235-239
61. Jain, M., Arvanitis, C., Chu, K., Dewey, W., Leonhardt, E., Trinh, M., Sundberg, C. D., Bishop, J. M., and Felsher, D. W. (2002) Science 297, 102-104
FIG. 1. Transcriptional repression of c-myc gene by SNP. A, P19 cells were treated with ActD (2 μg/ml) or CHX (2 μg/ml), for 15 or 30 min, respectively, prior to the addition of 0.5 mM SNP for 6 h. The c-myc mRNA was analyzed by an RT-PCR and the statistics of relative intensities of the amplified fragments were obtained from three experiments. B, P19 cells were treated with ActD (2 μg/ml) at different time points in the absence or presence of 1 mM SNP. The relative intensity of c-myc fragment was plotted against the time of incubation. The statistics were derived from three experiments.

FIG. 2. SNP-triggered decrease in DNA binding activity of NF-κB to the c-myc promoter. A, The c-myc gene has two putative NF-κB binding sites, one in the upstream enhancer (URE) and another in the internal exon 1 (IRE). The underlined GG in URE were mutated to CC (mt-URE). B, Competition and supershift analyses of κB-DNA binding complexes from P19 cells were conducted as described in text. Lane 1 is the control nuclear extract. Cold URE, mt-URE, or nonspecific oligomers (lanes 2, 3, and 4, respectively) was used for competition and the antibodies against p65, p50, or Sp1 (lanes 5, 6, and 7, respectively) were used in supershift experiments. F is free probes. C, Nuclear extracts from P19 cells treated with 0, 0.3, 0.5, or 1.0 mM of SNP (lanes 1, 2, 3, and 4, respectively) for 6 h were analyzed using (32P)-labeled URE or IRE as probes.

FIG. 3. Inhibition of NF-κB transcription activity by SNP. A, P19 cells and COS-1 cells were transiently transfected with p(Igk)4-LUC and treated with SNP for 6 h. The relative luciferase activity (RLU) was determined by normalizing each luciferase reading to an internal control and expressed as the average ± SD from three experiments (*p<0.05 vs. control P19 cells). B, COS-1 cells were cotransfected with p65 expression vector pcDNA-His-p65 (0.1 μg) and treated with SNP for 6 h. RLU was expressed as the average ± SD derived from three experiments (*p<0.05 vs. p65 without SNP).

FIG. 4. Repression of c-myc expression and NF-κB activity through peroxynitrite. A, P19 cells were treated with SNP for 6 h (lane 2), or pre-treated with 5 mM DTT or 0.1 mM DFO (lanes 3 and 4) for 30 min prior to the addition of SNP. The
untreated P19 cells were used as a control (lane 1). The expressed c-myc mRNA was analyzed by RT-PCR. B, Whole cell lysates were extracted from P19 cells which were treated as indicated, and were analyzed on a Western blot by using an anti-c-Myc Ab. C, P19 cells were transiently transfected with the reporter p(Igk)4-LUC and treated as described in A. The RLU was expressed as the average ± SD derived from two experiments (*p<0.01 vs. control, **p<0.05 vs. SNP).

FIG. 5. **Changing patterns of DNA binding of HEK293 cell extract through peroxynitrite.** HEK293 cells were stimulated by 10 ng/ml TNF-α for 8 h to activate NF-κB (lane 2) and treated with 1 mM SNP alone or in the presence of 0.1 mM DFO (lanes 3 and 4, respectively) for 6 h. NF-κB dimer and p50 homodimer complexes from the nuclear extracts were detected by EMSA. The nuclear extracts stimulated with TNF-α were used in supershift assays using antibodies against p65, p50, or Sp1 (lanes 5, 6, and 7, respectively). The supershifted (ss) band is marked with an asterisk.

FIG. 6. **The role of HDACs in the suppressive effect of SNP on NF-κB activity.** P19 cells transiently transfected with p(Igk)4-LUC were treated with 200 nM or 500 nM trichostatin A (TSA) for 24 h, followed by incubation with different concentrations of SNP for 6 h prior to the collection of these samples. The RLU was expressed as the average ± SD derived from two experiments (*p<0.005 vs. control, \(^p=0.05\) vs. control, **p<0.05 vs. 500 nM TSA, ***p<0.01 vs. 1 mM SNP).

FIG. 7. **Recruitment of NF-κB and HDACs 1 and 2 to the endogenous c-myc promoter in P19 cells treated with SNP.** A, ChIP analysis of histone acetylation on the endogenous c-myc promoter. The upper panel shows primers for amplifying the enhancer (URE) (-1357 to -1160) and 3’UTR of the mouse c-myc gene. P19 cells untreated, treated with 1 mM SNP, or SNP plus 0.1 mM DFO for 6 h (lanes 2, 3, and 4, respectively) were cross-linked with 1% formaldehyde and ChIP assays were conducted using anti-acetylated histone H4 (AcH4). The precipitated DNA was amplified by PCR for URE region (middle panel) followed by Southern blot analyses. As a negative control, 3’UTR
was amplified (lower panel). B, ChIP assays were performed using anti-p65, anti-p50, anti-HDAC1, or anti-HDAC2 Abs (left panels). The right panel shows a comparison of ChIP on the 3'UTR. C, The dynamics of p65 association with, and histone acetylation of, the endogenous c-myc promoter. P19 cells were treated with 1 mM SNP and ChIP assays were conducted, as described in A and B, at different time points.
Park-Fig. 1
Park-Fig. 2
Park-Fig.3
**Figure 4**

Panel A: Western blot analysis showing expression levels of c-myc and actin under different conditions.

Panel B: Similar analysis for another set of conditions.

Panel C: Graphical representation of relative luminescence units (RLU) for control (Con) and treated samples with SNP, DTT, and DFO. Significant differences are indicated by * and **.
Park-Fig.5
RLU

Con

0.5 mM SNP

1.0 mM SNP

200 nM TSA

500 nM TSA

0.5 mM SNP + 500 nM TSA

1.0 mM SNP + 500 nM TSA

1.0 mM SNP + 200 nM TSA

Park-Fig. 6
Park-Fig. 7

A

-1357 -1160

URE IRE

P1 P2

3' UTR

ChIP Ab

Con SNP SNP + DFO Con

Input 1 2 3 4 5 6

[]-AcH4

URE 3' UTR

B

ChIP Abs

Con SNP

Input []-p65 []-p50

Con SNP

Input []-HDAC1 []-HDAC2

E [] 3' UTR

C

Time of SNP treatment (min)

0 30 60 90 120

Input []-p65 []-AcH4
Regulation of c-myc gene by nitric oxide via inactivating NF-kB complex in P19 mouse embryonal carcinoma cells

Sung Wook Park and Li-Na Wei

J. Biol. Chem. published online June 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303306200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2003/06/03/jbc.M303306200.citation.full.html#ref-list-1