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Alteration of NF-κB p50 DNA Binding Kinetics by S-Nitrosylation

Andrew DelaTorre, Rebecca A. Schroeder, and Paul C. Kuo[1]
Department of Surgery, University of Maryland Medical Center, Baltimore, Maryland 21201

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Nitric oxide (NO) regulates a wide variety of cellular functions, in part, by formation of S–NO bonds at critical active site thiol groups within proteins, including transcription factors. Previous studies have qualitatively demonstrated that S-nitrosothiol formation can alter transcription factor binding to the DNA recognition site. To more precisely define the effect of S-nitrosylation on transcription factor binding, the equilibrium binding constant was derived for S-nitrosylated NF-κB p50 (S-NO-p50) in a cell free system utilizing gel shift assays. Binding of NF-κB p50 subjected to the nitrosylation conditions in the absence of $NaNO_2$ (C-p50-2) was not different from that of wild type NF-κB (C-p50-1). The extent of S-NO-p50 binding to its DNA target sequence was significantly decreased in comparison to that noted with C-p50-1 and C-p50-2. The binding constant was derived for each of the NF-κB variants: C-p50-1 = 1.01×10^{10} M^{-1}; C-p50-2 = 0.92×10^{10} M^{-1}; and S-NO-p50 = 0.28×10^{10} M^{-1}. These data indicate that S-nitrosylation of p50 decreases its affinity for the target DNA sequence by four-fold.

Key Words: **nitric oxide; transcription factor; promoter.**

Nitric oxide (NO) is a multifunctional free radical which regulates a wide variety of cellular functions, including inhibition of ribonucleotide reductase, activation of guanylyl cyclase, and inhibition of electron chain transport, and activation of $p21^{ras}$.(1,2) The broad range of bioregulatory targets for NO is, in large part, the result of its participation in redox chemistry. Regulation of protein function by the formation of S-nitrosothiols at critical active site thiol residues has been demonstrated to result in functional inhibition as exemplified by cathepsin B, aldolase, and aldehyde dehydrogenase.(1) Recently, transcription factors have been examined as potential bioregulatory targets for NO through formation of S-nitrosothiols. In cultured mouse cerebellar granule cells, Tabuchi and colleagues found that AP-1 DNA binding was inhibited in the presence of the NO donor, sodium nitroprusside.(3) Similarly, Park and coworkers have shown that formation of the NF-κB DNA complex was inhibited in the presence of the NO donor, spermine NONOate.(4) Additional studies by Matthews et al. found that S-nitrosylation of the redox sensitive NF-κB p50 C62 residue was associated with the inhibition of p50 binding to its consensus DNA target sequence.(5) These qualitative studies indicate that cellular NO production with subsequent S-nitrosylation of key transcription factors may serve as a regulator of transcription. However, effect of S-nitrosylation on the kinetics of transcription factor DNA binding has not been addressed. In this study, the effect of S-nitrosylation of human recombinant NF-κB p50 on its DNA equilibrium binding constant was analyzed.

MATERIALS AND METHODS

Materials. Human recombinant NF-κB p50 and Gel Shift Assay System, containing the NF-κB consensus oligonucleotide 5′-CGC-TTGATGAGTCAGCCGGAA-3′, were obtained from Promega Corp, Madison, WI. The DIG Gel Shift kit was purchased from Boehringer Mannheim Inc., Indianapolis, IN. All other chemicals were of reagent grade.

Synthesis of S-nitroso-p50. S-nitroso-p50 (S-NO-p50) was synthesized by combining equimolar $NaNO_2$ and p50 in 0.5N HCl for 30 minutes at room temperature as previously described. Before use, the S-nitrosoprotein solution was neutralized to pH 7.0 with 0.1N NaOH. Previous work has confirmed the presence of *S*-nitrosothiol bonds in the above species using ^{15}N-nuclear magnetic resonance spectroscopy.(6) Recombinant p50 (C-p50-1) and p50 subjected to the above reaction in the absence of $NaNO_2$ (C-p50-2) served as Controls.

Confirmation of S-nitrosylation of p50. The formation of an S-NO bond and its stability were validated by three principal analytical methods. The colorimetric assay using diazotization of sulfanilamide and subsequent coupling to N-(1-naphthyl) ethylenediamine was used to determine the concentration of nitrosating equivalents. S-nitrosothiol content of p50 was determined by monitoring the increase in A_{580} after selective displacement of the No group from S-nitrosothiols by Hg^{2+}. Confirmatory evidence for S-nitrosothiol bond

[1] Address all correspondence to Paul C. Kuo, 29 S. Greene St., #200, Baltimore, MD 21201. Fax: (410) 329-3837. E-mail: pkuo@surgery1.ab.umd.edu.

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formation was obtained by UV-visible spectrophotometry utilizing the absorption maxima for S-NO bonds at ~320-360 nm.(1)

Gel shift assay. Binding of recombinant NF-KB proteins to digoxigenin-labelled DNA containing an NF-KB recognition site was assayed by electrophoresis on 6% non-denaturing polyacrylamide (44:0.8 acrylamide: bisacrylamide) gels. Typically, 0.7 ng (0.018 pmol) recombinant p50 protein (diluted in PBS, 1 mg/ml BSA) was added without reducing agents to 17 μl of binding buffer (85 mM NaCl, 8.5% v/v glycerol, 22 mM HEPES pH 8.0, 1.3 mg/ml BSA, 0.17% NP-40, 3.6 mM spermidine, 0.85 mM EDTA pH 8.0, 6.1 mM $MgCl_2$). Digoxigenin-labelled double-stranded 21mer κB motif oligonucleotide (5′-CGCTTGATGAG TCAGCCGGAA-3′; typically 0.05 pmol) was added and the binding mixture (total mixture 20 μl) incubated for 15 min at 20°C before electrophoresis at 200 V for 40 min in 0.5x TBE buffer. After electrophoresis, transfer was performed onto nylon membrane. Detection was performed using a chemiluminescent detection technique based upon an alkaline phosphatase-linked anti-digoxigenin antibody. (DIG Gel Shift kit, Boehringer Mannheim Inc.).(7)

Determination of equilibrium binding constants. Binding of S-NO-p50 to labeled DNA fragments was determined by the gel electrophoresis assay. Complexes were formed at increasing concentrations of MLTF, establishing the equilibrium P + A ⇒ PA in which P is free DNA, A is free S-NO-p50, and PA is protein DNA complex (assuming a 1:1 binding stoichiometry). If total DNA is in vast molar excess over total MLTF in the reaction, it may be shown that

$$[PA] = \frac{K_{eq}[P_T]}{1 + K_{eq}[P_T]} \cdot [A_T]$$

where $[P_T]$ and $[A_T]$ are total DNA and S-NO-p50 concentrations, respectively, and K_{eq} is the apparent equilibrium binding constant. The concentration $[A_T]$ was determined by the method of Riggs et al., by measuring the concentration of protein-DNA complex when all of the S-NO-p50 was saturated with DNA.(8) The concentration of complex gives the concentration of active S-NO-p50. Active S-NO-p50 was then titrated against a 100-fold molar excess of DNA. The slope of the curve obtained by plotting complex concentration against total S-NO-p50 concentration was then used with the known $[P_T]$ to calculate K_{eq}. The procedure was then repeated for C-p50-1 and C-p50-2. (9)

Data analysis. Data are presented as mean ± SEM of four experiments.

RESULTS

Confirmation of S-Nitrosylation of NF-κB p50

The presence of S-NO bond formation in S-NO-p50 was confirmed by UV-visible spectrophotometry utilizing the dual absorption maxima for S-NO bonds at ~320-360 nm and at ~ 550 nm. (Figure 1) Absorption peaks were noted at both 330 nm and 550 nm for S-NO-p50. In contrast, there were no detectable peaks in the absorption spectra for either C-p50-1 or C-p50-2. In addition, Hg^{2+} displacement of NO from S-NO-p50 prepared in the presence of 1-, 10- and 100-fold excess $NaNO_2$ demonstrated that the stoichiometry of S-nitrosylation was 0.95 ± 0.1 (mol/mol) (n=5) and that the reaction was essentially complete after 30 minutes. The half-life for S-NO-p50 was ~8.5 hours in aqueous solution at pH 7.4. Previous work has confirmed the presence of *S*-nitrosothiol bonds in the above species using

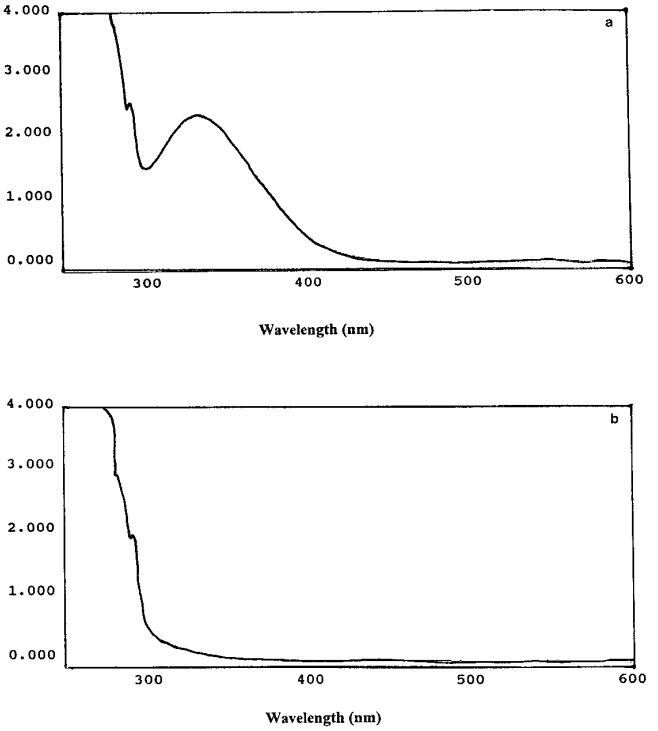

FIG. 1. UV-Visible spectroscopic characterization of S-nitrosylated NF-κB p50. $NaNO_2$ alone does not exhibit absorption at ~320–360 nm. (a) S-nitrosation of NF-κB p50 with 10-fold molar excess of acidified $NaNO_2$. (b) Wild type NF-κB p50.

^{15}N-nuclear magnetic resonance spectroscopy. (Data not shown.)

Gel Shift Assays of S-NO-p50, C-p50-1 and C-p50-2

Gel shift assays were performed using S-NO-p50, C-p50-1 and C-p50-2. (Figure 2.) The extent of S-NO-p50 binding to its DNA target sequence was significantly decreased in comparison to that noted with C-p50-1 and C-p50-2. Binding of NF-κB p50 subjected to the nitrosylation conditions in the absence of $NaNO_2$ (C-p50-2) was not different from that of wild type NF-κB (C-p50-1). K_{eq} was then calculated for each of the NF-κB variants: C-p50-1 = 1.01×10^{10} M^{-1}; C-p50-2 = 0.92×10^{10} M^{-1}; and S-NO-p50 = 0.28×10^{10} M^{-1}. (Figure 3.) These results indicate that S-nitrosylation of NF-κB p50 is associated with decreased affinity for its DNA target sequence as evidenced by the four-fold decrease in K_{eq}.

DISCUSSION

In this study, we demonstrate that S-nitrosylation of NF-κB p50 is associated with a four fold decrease in its equilibrium binding constant for its consensus oligonucleotide response element. The binding constant associated with p50 protein which underwent the nitrosy-

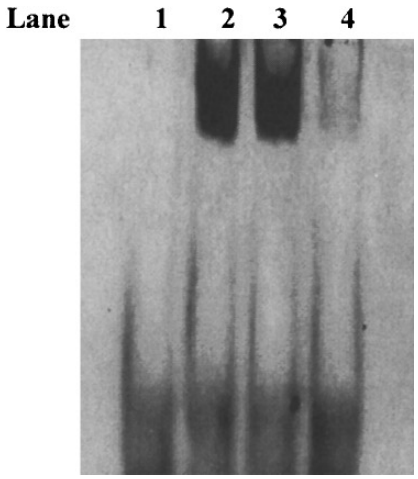

FIG. 2. Effect of S-nitrosylation on NF-κB p50 DNA binding. Recombinant p50 protein (diluted in PBS, 1 mg/ml BSA) was added without reducing agents to 17 μl of binding buffer (85 mM NaCl, 8.5% v/v glycerol, 22 mM HEPES pH 8.0, 1.3 mg/ml BSA, 0.17% NP-40, 3.6 mM spermidine, 0.85 mM EDTA pH 8.0, 6.1 mM $MgCl_2$). Digoxigenin-labelled double-stranded 21mer κB motif oligonucleotide (5′-CGCTTGATGAG TCAGCCGGAA-3′; typically 0.05 pmol) was added and the binding mixture (total mixture 20 μl) incubated for 15 min at 20°C before electrophoresis at 200 V for 40 min in 0.5x TBE buffer. After electrophoresis, transfer was performed onto nylon membrane. Detection was performed using a chemiluminescent detection technique based upon an alkaline phosphatase-linked anti-digoxigenin antibody. Lane 1, Oligonucleotide probe alone. Lane 2, C-p50-1 + probe. Lane 3, C-p50-2 + probe. Lane 4, S-no-p50 + probe. Gel is representative of four experiments.

lation reaction in the absence of $NaNO_2$ was not significantly different than that of wild type p50. This indicates that the conditions associated with the nitrosylation reaction were not responsible for the altered K_{eq} associated with S-NO-p50. In addition, the stoichiometry of the reaction suggests the presence of only a single S-NO bond in S-NO-p50. This is surprising given the presence of 13 potential thiol groups in the primary amino acid sequence of NF-κB p50. Our results suggest that S-nitrosylation of transcription factor proteins with subsequent alteration in DNA binding kinetics represents a mechanism by which NO exerts its regulatory functions.

In recent years, NO has emerged as pleuripotent regulatory free radical with multiple physiologic roles. In 1992, Stamler and colleagues suggested that S-nitrosothiol groups in proteins may serve as intermediates in the cellular metabolism of NO and as NO donors which extend the functional half-life of NO.(6) Subsequently, S-nitrosylation has been demonstrated in the altered function of multiple proteins: tissue-type plasminogen activator, cathepsin B, glyceraldehyde-3-phosphate dehydrogenase, charybdotoxin-sensitive potassium channels, $p21^{ras}$ and O^6-methylguanine-DNA-methyl transferase.(1) These observations recently have been extended to include nuclear protein transcription factors, including AP-1 and NF-κB.(3-5) Peng and coworkers demonstrated that NO donors inhibited NF-κB activation by tumor necrosis factor-α in human vascular endothelial cells. They implicated induction and stabilization of IκBα by NO as the underlying mechanism.(10) In contrast, Matthews and colleagues showed that NO donors inhibited NF-κB DNA binding by S-nitrosylation of the redox sensitive C62 residue.(5) In similar studies using the transcription factor, AP-1, Tabuchi and coworkers demonstrated the inhibition of AP-1 DNA binding in the presence of NO donors. The mechanism was not addressed in this study, although the authors suggest S-nitrosylation of AP-1 as a potential explanation.(3) These studies demonstrated that NO can inhibit the binding of NF-κB p50 to its response element. One mechanism acts via S-nitrosylation of key thiols which maintain the appropriate conformation of the DNA binding site or tertiary structure of p50. Alternatively, S-nitrosylation may interfere with binding itself by steric hindrance or chemical alteration of key residues participating in DNA binding. These data are particularly relevant given the recent discovery by Park and colleagues that NO autoregulates iNOS gene transcription by inhibiting DNA binding of NF-κB.(4)

However, these previous studies have only addressed the question in a qualitative fashion. In this study, we have determined the effect of S-nitrosylation on the K_{eq} for p50 binding to its response element. S-nitrosylation of p50 resulted in a four-fold decrease in the equilibrium binding constant. If inhibition of NF-κB binding *in vitro* occurs as the result of both IκBα stabilization and inhibition of p50 binding, then this four fold decrease in binding affinity will be increased in a multiplicative fashion.

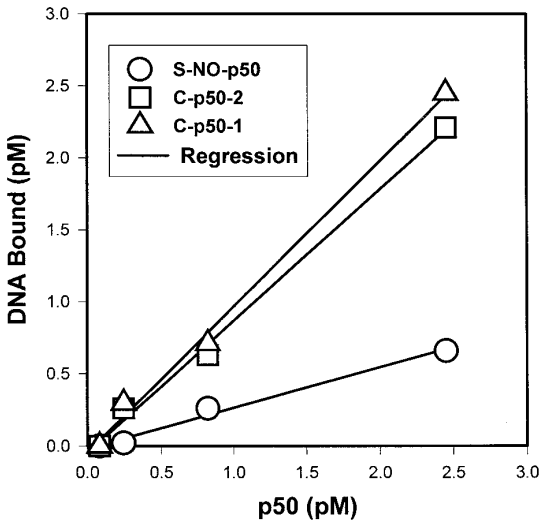

FIG. 3. Equilibrium binding reaction of C-p50-1, C-p50-2, and S-NO-p50. Reactions were performed at a temperature of 30 °C in the presence of 5 mM $MgCl_2$.

NO is a multifunctional regulatory messenger. It has been found to modulate numerous cellular and biochemical functions. However, many of these observations are based upon global inhibition of NO synthesis by administration of competitive substrate inhibitors. The specific molecular mechanisms inducing NO production and modulatory functions are largely unknown. Recently, the interaction of NO with protein thiol groups has been noted to significantly alter protein function. Specifically, DNA binding activity of NF-κB can be modified by S-nitrosylation. In this study, we demonstrate that S-nitrosylation occurs at a single thiol residue and is associated with a significant decrease in DNA binding as measured by the equilibrium binding constant. Although these data are derived from a cell-free system, they mirror qualitative cellular findings.

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