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Inhibition of NF-κB DNA binding by nitric oxide

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ABSTRACT

It has been suggested that the NF-κB transcription factor family may mediate expression of the gene encoding the cytokine-inducible form of nitric oxide synthase (iNOS). To establish if nitric oxide (NO) could in turn affect activity of NF-κB, the ability of NO-donor compounds to influence NF-κB DNA binding activity *in vitro* was investigated. NO-donor compounds sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) both inhibited the DNA binding activity of recombinant NF-κB p50 and p65 homodimers and of p50–p65 heterodimers. Inhibition of NF-κB p50 DNA binding by NO-donor compounds involved modification of the conserved redox-sensitive C62 residue, as a C62S p50 mutant was significantly more resistant to SNP-mediated inactivation. Non-reducing SDS–polyacrylamide gel electrophoresis demonstrated that SNP could inhibit p50 DNA binding by mechanisms other than the formation of intersubunit disulphide bonds involving p50 residue C62. Electrospray ionization mass spectrometry of a synthetic NF-κB p50 peptide containing the C62 residue suggested that NO gas can modify C62 by S-nitrosylation. This study indicates that NO-donors can directly inhibit the DNA binding activity of NF-κB family proteins, suggesting that cellular NO provides another control mechanism for modulating the expression of NF-κB-responsive genes.

INTRODUCTION

The physiological roles of the short-lived free radical gas nitric oxide (NO) have been the subject of intense interest since the identification of NO as the diffusible agent mediating the relaxation of smooth muscle (1), reviewed in (2–5). NO has subsequently been shown to be involved in a wide range of biological processes including vasodilation, platelet aggregation, macrophage cytotoxicity and neurotransmission.

It has been demonstrated that treatment of cells with NO-generating compounds such as sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP) and S-nitrosoglutathione (GSNO) influences activation of the nuclear factor κB (NF-κB)/rel/dorsal family reviewed in (6,7). An early study in normal human peripheral blood mononuclear cells suggested that treatment with NO-donors SNP and SNAP resulted in the activation of the DNA binding activity of NF-κB family proteins (8). In contrast, in more recent studies using human vascular endothelial cells, activation of NF-κB DNA binding activity following treatment with tumour necrosis factor-α (TNF-α) could be inhibited by treatment with SNP and GSNO (9). It was proposed that the mechanism of inhibition by NO-donors involved both stabilization of IκBα, and increased transcription of the IκBα gene (10).

Inducibility of the DNA binding activity and nuclear translocation of NF-κB, whose classical form is a heterodimer of p50 and p65 subunits, is mediated by the disruption of complexes of NF-κB and IκB inhibitor proteins. In unstimulated cells NF-κB family proteins are normally held in a non DNA-binding form in the cytoplasm by IκB proteins. Stimulation of cells with a wide range of agents results in proteolytic degradation of IκBα, allowing the nuclear translocation of NF-κB, now in a form competent for DNA binding. NF-κB family proteins recognize DNA sequences related to the κB motif (5′-GGGACTTTCC-3′). Similar DNA sequences are present in transcriptional control regions of cellular genes involved in immune and inflammatory responses, and are important for transcription of a number of viral genes, such as the human immunodeficiency virus (HIV) provirus (reviewed in 6,7).

Members of the NF-κB transcription factor family include the p50 (NF-κB1), p52 (NF-κB2), p65 (Rel A), Rel B, c-Rel, v-Rel, dorsal and Dif proteins. These NF-κB family proteins share a conserved N-terminal ~300 amino acid region known as the NF-κB/rel/dorsal (NRD) homology region which is responsible for DNA binding, dimerization and nuclear localization (6,7). Amino acid residues in the N-terminal part of the NRD region contribute to specific DNA recognition and are responsible for redox modulation of DNA binding activity (11–16). The κB motif DNA is recognised in an unusual way, with the NF-κB making base and backbone contacts with the DNA over one complete turn of the double helix (17,18). Structural analysis of p50 homodimer–κB motif DNA co-crystals (19,20) has revealed that the p50 monomer contains two domains separated by a potentially flexible linker. The N-terminal domain is responsible for most of the specific DNA contacts, while the C-terminal domain forms the dimer interface but also contacts κB motif DNA.

It has been suggested that reactive oxygen species such as hydroxyl radicals and superoxide anions act as mediators of

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NF-κB activation by IκB degradation (21,22) although NF-κB must be in a reduced state to bind DNA *in vitro* (23). This effect is mediated by the redox state of a cysteine residue conserved in all NF-κB/rel/dorsal subunit species, corresponding to C62 in the NF-κB p50 subunit (11–15). The C62 residue is located within a polypeptide loop used to make many of the specific contacts with κB motif DNA, and contacts a phosphate(s) in the DNA backbone (19,20).

Following activation of NF-κB DNA binding activity and NF-κB translocation to the nucleus, a mechanism is required to shut off expression of NF-κB-responsive genes to allow the cell to return to its initial state. This could be achieved by exporting NF-κB from the nucleus back to the cytoplasm, a process recently shown to be mediated by IκBα (24), and/or by inactivating nuclear NF-κB.

The promoter of the murine inducible nitric oxide synthase (iNOS) gene contains two κB motifs, one of which is critical for inducible gene expression (25). Similarly, several cytokine-responsive elements are present in the human iNOS gene promoter, including a κB motif, three interferon-γ response elements and a tumour necrosis factor response element (26), suggesting an important role for NF-κB in NO production. NO has also been shown to inactivate several proteins by modification of reactive thiol groups (27) and mass spectroscopic analysis has shown that NO gas can modify cysteine groups by S-nitrosylation (28). As members of the NF-κB/rel/dorsal family all posess a reactive redox-sensitive cysteine residue towards the N-terminus of their NRD region, this study explored the possibility of a direct effect of NO on NF-κB DNA binding activity using recombinant NF-κB p50 and p65 proteins.

MATERIALS AND METHODS

Bacterial expression and purification of NF-κB proteins

NF-κB p65 (residues 12–317), NF-κB p50 (residues 35–381) and a C62S p50 mutant were expressed in *Escherichia coli* JM101. The wild-type p50 and C62S mutant p50 proteins were purified as described previously (12), p65 protein was purified similarly. Protein fractions were frozen in liquid nitrogen and stored at –70°C. Protein purity was determined by analysis under reducing conditions in 10% polyacrylamide gels containing SDS.

Gel electrophoresis DNA binding assay

Binding of recombinant NF-κB proteins to ^{32}P-labelled DNA containing an NF-κB 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, 0.62 ng (0.018 pmol) recombinant p65 protein or 0.35 ng of recombinant p50 plus 0.31 ng of recombinant p65 (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 $MgCl_2$). If needed, reducing agents were added at this point and the mixture incubated on ice for 15 min. Where indicated, NO-generating agents or other reagents were then added and the mixture incubated on ice [15 min for SNP (Sigma) or 1 h for SNAP (Sigma)]. Finally, 5′-^{32}P-labelled double-stranded 16mer κB motif oligonucleotide (5′-CTGGGGACTTTCCAGG-3′, typically 0.05 pmol) was added and the binding mixture (total volume 20 µl) incubated for 15 min at 20°C before electrophoresis at 200 V for 40 min in 0.5× TBE buffer. After electrophoresis, gels were dried on DEAE–cellulose paper (Whatman, DE81) and visualised by autoradiography.

Non-reducing SDS–polyacrylamide gel electrophoresis/western blotting

To detect intersubunit disulphide crosslinks involving C62 thiol groups of p50 homodimers (or other possible covalent intersubunit crosslinks), p50 proteins were analysed by electrophoresis in the presence of SDS on non-reducing 10% polyacrylamide gels (with 29:1 acrylamide:bisacrylamide). Following electrophoresis, SDS–protein complexes were electroblotted (LKB semi-dry blotter, with 20 mM Tris, 150 mM glycine, 20% methanol transfer buffer) onto polyvinylidene difluoride (PVDF, Sigma) membrane. PVDF membranes were blocked for 1 h in PBS, 0.1% Tween-20, 10% non-fat dry milk (PTM) before incubating for 1 h at room temperature with polyclonal anti-p50 antiserum diluted 1:1000 with PTM. Membranes were again washed with PTM and incubated with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham, diluted 1:500 in PTM) for 30 min at room temperature. Finally, PVDF membranes were washed twice with PTM, once with PBS, 0.1% Tween-20 before incubating with enhanced chemiluminescence detection reagent (Amersham) and detection with X-ray film.

Measurement of free NO concentrations

NO concentration measurements were performed in 80 ml of DNA binding assay binding buffer in an argon-blanketed sealed container at room temperature using an isolated nitric oxide meter and probe (World Precision Instruments Iso-NO, previously calibrated with NO gas) with 3 min intervals between SNP or SNAP sample additions.

Mass spectrometry of p50 peptide after NO gas treatment

An oligopeptide corresponding to p50 amino acid residues 43–77 and of predicted average molecular mass 3933.47 Da was synthesised and then purified by reverse phase HPLC. The integrity of the peptide was confirmed by N-terminal amino acid sequencing. The p50 peptide was analysed by electrospray ionization mass spectrometry in the positive ion mode using a VG Quattro instrument (VG Organic, Altrincham, UK) previously calibrated with myoglobin. Nitric oxide gas was generated by the reaction of ascorbic acid with sodium nitrite in solution under a nitrogen atmosphere. The gas generated was passed through a sodium hydroxide scrubbing solution to remove NO_2 before being passed via a Pasteur pipette at ~20 ml/min for ~5 min into 0.15 mg of reduced p50 peptide dissolved in 50 µl of ultrapure water:methanol:acetic acid (1:1:0.01). Samples of the peptide solution (5 µl) were analysed by electrospray ionization mass spectrometry before and ~20 min after the NO gas treatment.

RESULTS

Inactivation of NF-κB DNA binding by NO-donors

To investigate direct effects of NO on NF-κB DNA binding activity, purified recombinant p50 homodimers were incubated with NO-donor compounds in the presence or absence of DTT and the DNA binding activity determined in a gel electrophoresis

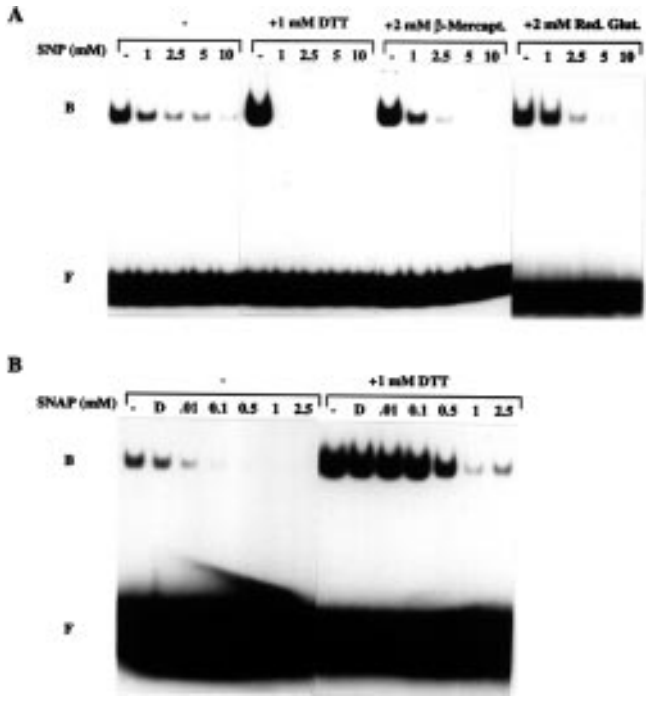

Figure 1. Inhibition of NF-κB p50 DNA binding activity by the NO-donors SNP and SNAP. (**A**) No addition, or addition of DTT to 1 mM, or addition of β-mercaptoethanol to 2 mM, or addition of reduced glutathione to 2 mM to 1.4 ng (0.036 pmol) of recombinant p50 protein [or 0.7 ng of recombinant p50 (0.018 pmol) in the reduced glutathione case] in 17 µl of binding assay buffer, followed by addition of SNP to the indicated concentrations. (**B**) No addition, or addition of DTT to 1 mM to 0.7 ng (0.018 pmol) of recombinant p50 protein in 17 µl of binding assay buffer, followed by addition of 1 µl of SNAP stock solution in DMSO to give the indicated SNAP concentrations. DMSO (1 µl) was added to lanes marked 'D' as a control for effects on DNA binding activity. The positions of DNA–protein complexes and free oligonucleotide probe are indicated by 'B' and 'F' respectively.

DNA binding assay using a ^{32}P-labelled DNA oligonucleotide containing the recognition site for NF-κB. While SNP in the absence of DTT inhibited p50 DNA binding in a gradual manner, SNP in combination with 1 mM DTT resulted in a dramatic inhibition of p50 DNA binding activity (Fig. 1A). SNP in combination with 2 mM β-mercaptoethanol or with the physiological antioxidant reduced glutathione (at 2 mM) also potentiated the inhibitory effect of SNP on p50, although again not as effectively as 1 mM DTT (Fig. 1A). The dramatic inhibition of NF-κB p50 DNA binding activity by the combination of SNP and DTT was not due to any inhibitory effect of DTT itself as reducing agents stimulate the DNA binding activity of oxidised native and recombinant NF-κB proteins (11–15,23). Comparison of the two control lanes (±1 mM DTT) in the absence of SNP clearly illustrates this point (Fig. 1A).

To confirm that the inhibition of NF-κB p50 DNA binding activity by SNP was a consequence of NO release, a chemically distinct NO-donor was tested. S-nitroso-N-acetylpenicillamine (SNAP) was incubated with NF-κB p50 in the presence or absence of reducing agent and the DNA binding activity remaining determined in a gel electrophoresis DNA binding assay. Addition of SNAP to the DNA binding assay gave similar results in either the presence or absence of 1 mM DTT, with 1 mM SNAP reducing p50 DNA binding activity to undetectable levels (Fig. 1B).

To exclude the involvement of other species in the SNP (plus DTT)-mediated inhibition of NF-κB p50 DNA binding activity, CN^-, NO_2^- and NO_3^- [which are also formed during or after the reaction of SNP with DTT (29)] were added to the DNA binding assay. The addition of KCN, $NaNO_2$ or $NaNO_3$ had no significant inhibitory effect on p50 DNA binding activity when added to the binding assay buffer in either the absence or presence of 1 mM DTT (data not shown). Further, the addition of potassium ferrocyanide which is structurally similar to SNP but lacks any NO groups had no significant effect on p50 DNA binding activity either in the absence or presence of 1 mM DTT (data not shown).

To determine if NO-donor compounds could inhibit the DNA binding activity of other NF-κB species, recombinant p65 homodimers were treated with SNP or SNAP in the presence or absence of 1 mM DTT. Treatment of p65 with 1 mM DTT stimulated p65 DNA binding activity, and again the combination of SNP plus DTT was a very effective inhibitor of p65 homodimer DNA binding (Fig. 2A). Treatment of p65 with SNAP resulted in complete inhibition of p65 DNA binding activity by 1 mM SNAP (Fig. 2B). Similarly, the DNA binding activity of the classical p50–p65 heterodimer form of NF-κB was reduced to undetectable levels by treatment with 1 mM SNP plus DTT (Fig. 3A) or by 1 mM SNAP (Fig. 3B).

Site of inactivation of p50 by the NO-donor SNP

As several studies had previously identified the critical role of C62 (which is conserved throughout the NF-κB/rel/dorsal family) in regulating the DNA binding activity of p50, the effect of SNP on the DNA binding activity of a C62S mutant of p50 (12) was examined. While the wild-type p50 DNA binding activity was completely inhibited by 1 mM SNP in the presence of DTT (Fig. 4A), the C62S mutant was considerably more resistant to inactivation by SNP either in the absence or presence of DTT (Fig. 4B). This result implicates the redox-sensitive C62 residue of p50 as the target of inactivation by SNP.

Free NO concentrations generated by NO-donors

As the concentrations of both SNP and SNAP needed to inhibit NF-κB DNA binding activity *in vitro* were relatively high compared with estimated physiological NO concentrations, the free NO concentration in the gel electrophoresis DNA binding assay binding buffer after NO-donor addition was measured using an NO-sensitive probe (World Precision Instruments Iso-NO). The free NO concentrations as measured by the probe were several orders of magnitude lower than the concentrations of SNP and SNAP (Fig. 5A and B), suggesting that these *in vitro* phenomena are likely to be physiologically relevant.

Nature of the SNP modification of p50 residue C62

Of the chemical modification events which could occur during SNP-mediated inhibition of p50 DNA binding, one possibility might be the formation of an intersubunit disulphide bond involving residue C62. It has been previously shown that in oxidised p50 homodimers, the inactivation of p50 DNA binding is mediated by the formation of an intersubunit disulphide bond involving C62, a residue known to make close contacts with κB motif DNA (11,12,15,19,20). Hence, NF-κB p50 protein was treated with SNP or the sulphydryl oxidising agent diamide (Azodicarboxylic acid bis [dimethylamide]) in the presence or

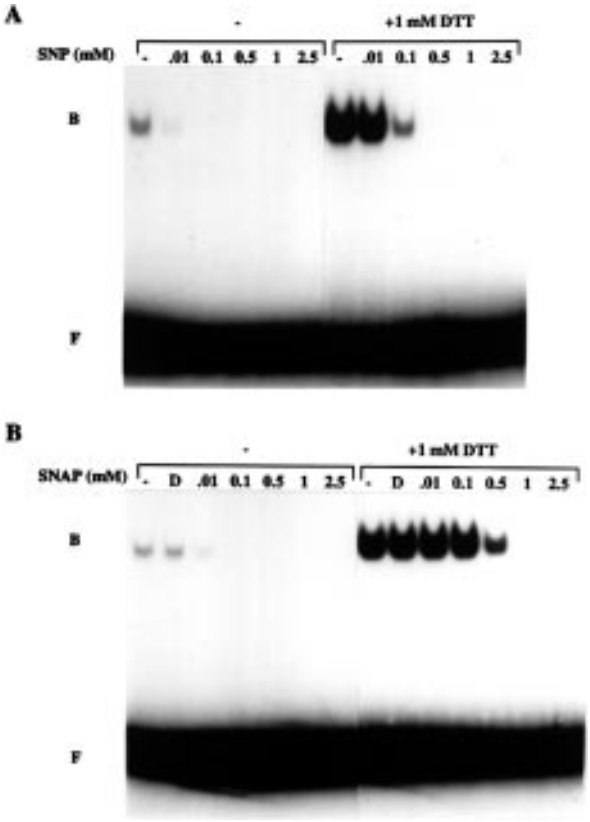

Figure 2. Inhibition of NF-κB p65 DNA binding activity by the NO-donors SNP and SNAP. (**A**) No addition, or addition of DTT to 1 mM to 0.62 ng (0.018 pmol) of recombinant p65 protein in 17 µl of binding assay buffer, followed by addition of SNP to the indicated concentrations. (**B**) No addition, or addition of DTT to 1 mM to 0.62 ng of recombinant p65 protein in 17 µl of binding assay buffer, followed by addition of 1 µl of SNAP stock solution in DMSO to give the indicated SNAP concentrations. DMSO (1 µl) was added to lanes marked 'D' as a control for effects on DNA binding activity. After incubation on ice, 0.05 pmol of κB motif oligonucleotide probe was added as normal. The positions of DNA–protein complexes and free oligonucleotide probe are indicated by 'B' and 'F' respectively.

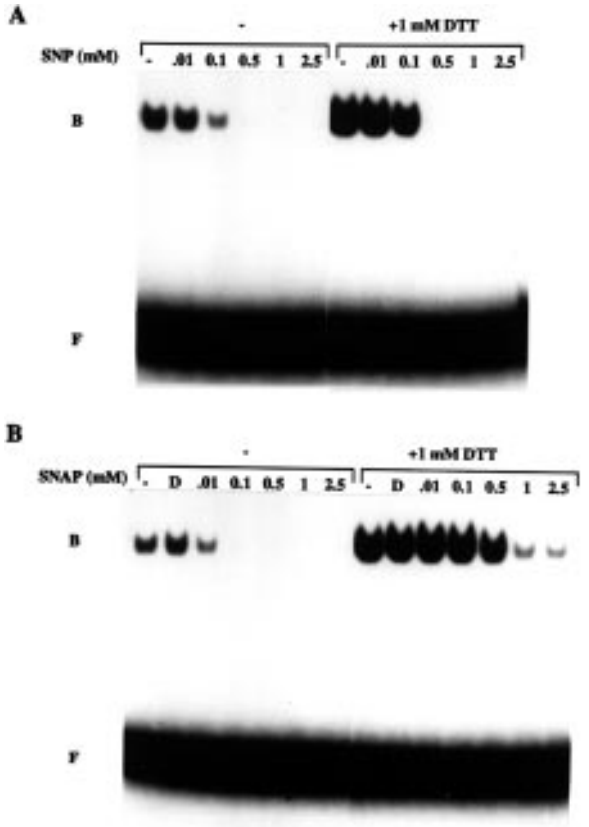

Figure 3. Inhibition of NF-κB p50–p65 heterodimer DNA binding activity by the NO-donors SNP and SNAP. (**A**) No addition, or addition of DTT to 1 mM to 0.35 ng (0.009 pmol) of recombinant p50 protein plus 0.31 ng (0.009 pmol) of recombinant p65 protein in 17 µl of binding assay buffer, followed by addition of SNP to the indicated concentrations. (**B**) No addition, or addition of DTT to 1 mM to 0.35 ng of recombinant p50 protein plus 0.31 ng of recombinant p65 protein in 17 µl of binding assay buffer, followed by addition of 1 µl of SNAP stock solution in DMSO to give the indicated SNAP concentrations. DMSO (1 µl) was added to lanes marked 'D' as a control for effects on DNA binding activity. After incubation on ice, 0.05 pmol of κB motif oligonucleotide probe was added as normal. The positions of DNA–protein complexes and free oligonucleotide probe are indicated by 'B' and 'F' respectively.

absence of 1 mM DTT, the reaction products analysed by non-reducing SDS–PAGE and correlated with their activity in a DNA binding assay.

The polyclonal anti-p50 western blot analysis of the non-reducing SDS–PAGE suggests that in the absence of 1 mM DTT, the majority of the SNP-treated p50 is non-covalently associated (although a significant amount of covalently linked p50 dimer and higher molecular weight forms are visible). Whereas essentially all of the diamide-treated p50 is present as covalently linked dimer and higher molecular weight forms in the absence of 1 mM DTT (Fig. 6A). The relatively weak chemiluminescence signals from the covalently linked p50 forms is probably due to poor transfer of these high molecular weight species from the polyacrylamide gel to the PVDF membrane. Comparison with the corresponding DNA binding assay reveals that, as expected, treatment of p50 with either SNP or diamide in the absence of DTT causes almost complete loss of DNA binding activity (Fig. 6B).

In contrast, in the presence of 1 mM DTT, both the SNP and diamide-treated p50 proteins are non-covalently associated as shown by non-reducing SDS–PAGE (Fig. 6A). As expected, the DNA binding assay shows that while p50 treated with a combination of diamide and a molar excess of DTT has fully regained its binding activity, the combination of SNP and 1 mM DTT has completely inhibited p50 DNA binding activity (Fig. 6B). Thus it seems that while p50 DNA binding inactivation by diamide operates by formation of an intersubunit disulphide bond involving residue C62 (this work and ref. 12), the NO-donor SNP (plus DTT) can inactivate NF-κB p50 DNA binding without the formation of any intersubunit covalent bond.

Mass spectrometric characterisation of the NO modification of p50

As the redox-sensitive p50 C62 residue has been implicated as the primary target of SNP-mediated inactivation of p50 DNA binding activity, a synthetic peptide corresponding to p50 residues 43–77 (of predicted average molecular mass 3933.47 Da) was studied by electrospray ionization mass spectrometry. Initially the mass of the p50 peptide in a water:methanol:acetic acid (1:1:0.01)

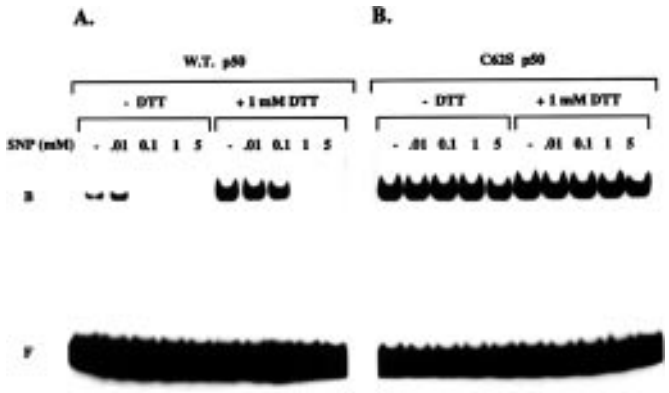

Figure 4. Demonstration of the lower sensitivity of a C62S p50 mutant towards inhibition of DNA binding activity by SNP. (**A**) Wild-type p50 protein (0.7 ng) was incubated in 17 µl of binding assay buffer with no addition of DTT, or addition of DTT to 1 mM. This was followed by addition of SNP to the indicated concentration. (**B**) C62S mutant p50 protein (3.9 ng) was incubated in 17 µl of binding assay buffer with no addition of DTT, or addition of DTT to 1 mM. This was followed by addition of SNP to the indicated concentration. The positions of DNA–protein complexes and free oligonucleotide probe are indicated by 'B' and 'F' respectively.

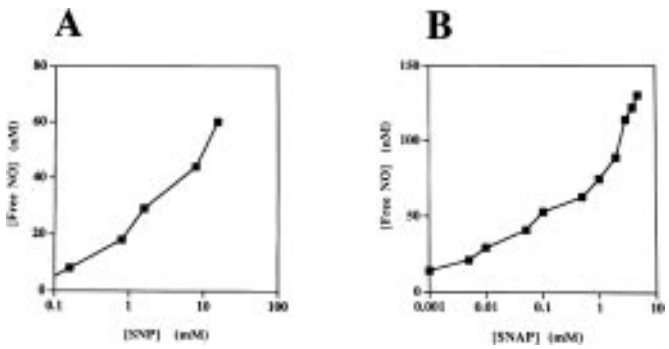

Figure 5. Measurements of free NO concentrations in DNA binding assay binding buffer following addition of NO-donor compounds. (**A**) NO meter readings (World Precision Instruments Iso-NO) taken 3 min after addition of SNP solution to 80 ml of DNA binding assay binding buffer supplemented with 1 mM DTT. (**B**) NO meter readings taken 3 min after addition of SNAP dissolved in DMSO to 80 ml of DNA binding assay binding buffer, performed in the absence of DTT.

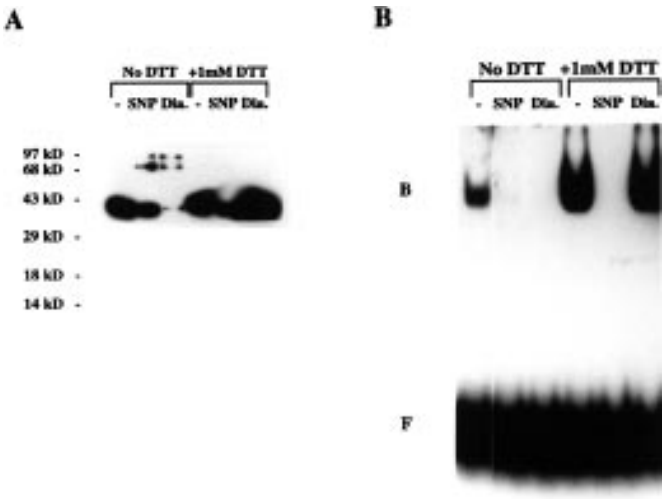

Figure 6. Correlation of SNP's effect on p50 DNA binding activity with the intersubunit association state of p50 homodimers, showing that SNP can inactivate p50 DNA binding without forming an intersubunit disulphide bond. p50 protein was reduced by addition of DTT to 25 mM, then the excess DTT removed by passage over a Biogel P6 column. Aliquots containing 80 ng of p50 protein in 40 µl of 25 mM HEPES pH 8.0, 1 mM EDTA pH 8.0, 0.05% NP-40, 10% glycerol, 100 mM NaCl, 0.1 mg/ml BSA buffer were either untreated or supplemented with DTT to 1 mM and incubated on ice for 15 min. Following this, aliquots were either left untreated, or SNP added to 10 mM or diamide added to 0.2 mM and incubated for a further 15 min on ice. (**A**) Fifteen microlitre samples (containing 30 ng p50) were removed from the 40 µl aliquots, 5 µl of sample buffer minus β-mercaptoethanol added, and the mixture heated to 100°C for 2 min before loading onto a 10% SDS–polyacrylamide gel and electrophoresis at 100 V. The mobilities of pre-stained molecular weight standards are indicated. (**B**) One microlitre samples (containing 2 ng p50) were removed from the 40 µl aliquots and added to 17 µl of 25 mM HEPES pH 8.0, 1 mM EDTA pH 8.0, 0.05% NP-40, 10% glycerol, 100 mM NaCl, 0.1 mg/ml BSA buffer and incubated 15 min on ice. Finally, 0.075 pmol of ^{32}P-radiolabelled 16mer κB motif oligonucleotide probe was added and the mixture incubated 15 min at 20°C before loading directly on the non-denaturing gel and electrophoresis at 200 V. The positions of DNA–protein complexes and free oligonucleotide probe are indicated by 'B' and 'F' respectively.

solution was measured following transformation from a mass/charge scale to a true molecular mass scale as 3933.5 Da (Fig. 7A). Following exposure to the NO gas stream for ~5 min, a significant fraction of the p50 peptide had been modified to yield a species of mass 3962.6 Da (Fig. 7B). This increase in mass of 29.1 Da would be consistent with the nitrosylation of the peptide with the loss of a hydrogen. A similar mass increase was seen following treatment of the p50 peptide with the NO-donor SNAP (data not shown).

DISCUSSION

As the common property of SNP and SNAP is their behaviour *in vivo* and *in vitro* as NO-donors, it seems likely that free NO or a closely related species such as NO^+ is directly involved in the *in vitro* modification of NF-κB proteins. This behaviour is similar to that seen with AP-1 transcription factor DNA binding activity in cerebellar granule cell nuclear extracts, in that SNP treatment inactivated AP-1 (29). Interestingly, AP-1 transcription factor DNA binding activity is also modulated by the redox state of a conserved cysteine residue in each subunit of the dimer (30).

Although the concentrations of SNP and SNAP needed to inhibit the DNA binding activity of NF-κB proteins *in vitro* in these studies were high (in the range of 0.1–1 mM) compared with normal physiological NO concentrations, ~10 nM in the brain (31), the concentrations of free NO as measured by the NO probe were much lower (in the range 20–100 nM) suggesting that the NO-donor inhibitory species could be present at physiologically-relevant concentrations. Although the exact nature(s) of the inhibitory species is uncertain, for the NO-donor SNP one candidate is NO^+ which, unlike NO, can readily react with thiolate groups to form RS-N=O (4,32).

Intriguingly, a recent report has shown that NO can regulate the reactivation of latent Epstein–Barr virus by down-regulation of the redox-sensitive viral transcription factor Zta. Since Zta can activate the expression of its own gene, it was suggested that NO

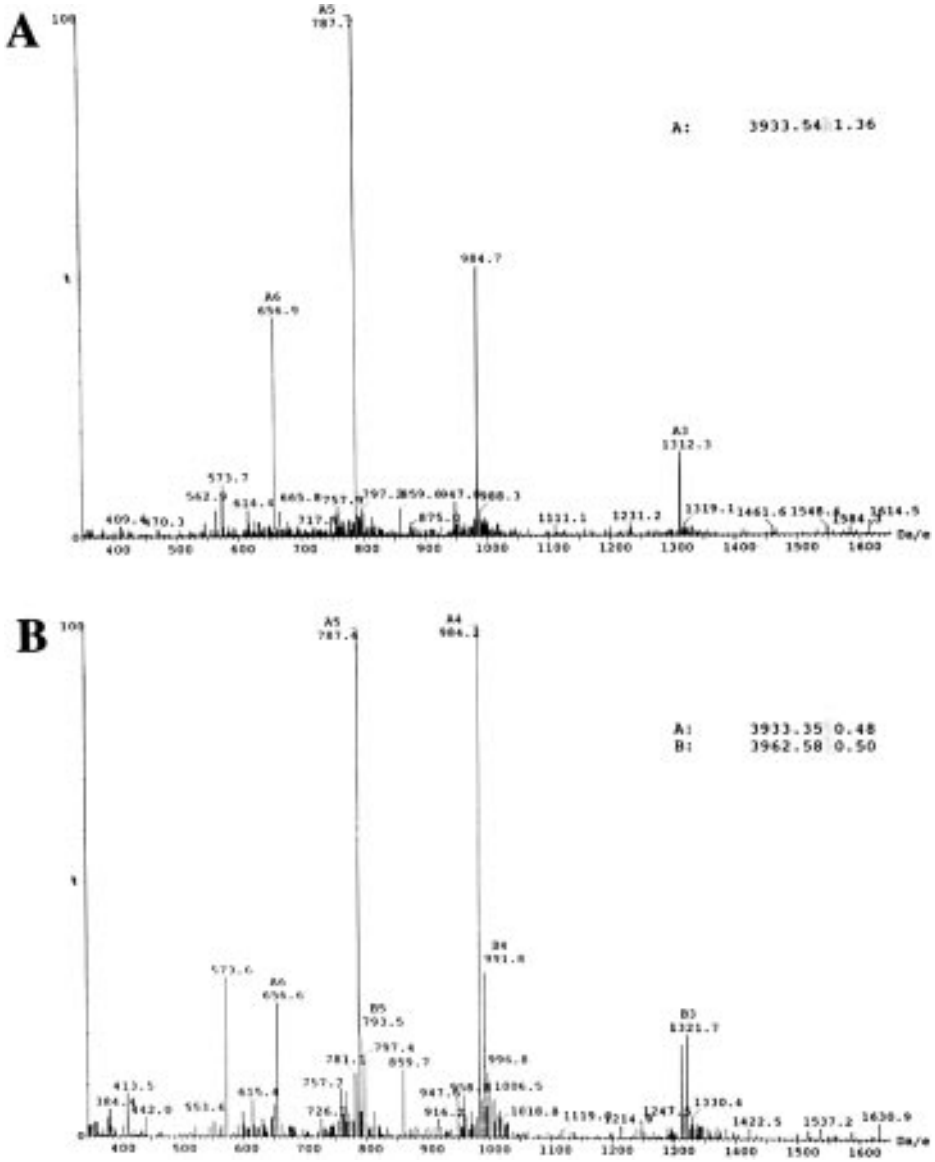

Figure 7. Electrospray ionization mass spectrometry of p50 amino acid residue 43–77 synthetic peptide before and after reaction with NO gas showing a 29.1 Da mass increase following NO treatment. (**A**) Original multiply charged mass/charge spectrum from 5 µl (15 µg peptide) of p50 peptide in ultrapure water:methanol:acetic acid (1:1:0.01) mixture. (**B**) Multiply charged mass/charge spectrum from 5 µl (15 µg peptide) of p50 peptide in ultrapure water:methanol:acetic acid (1:1:0.01) following NO gas saturation for ~5 min and mass spectrometric analysis after ~20 min.

could inhibit Zta function by reaction with its reactive cysteine residue(s) either through S-nitrosylation or by accelerating formation of an intersubunit disulphide bond (33).

The role of NO in modulating the activation of NF-κB *in vivo* remains confused. In normal human peripheral blood mononuclear cells, SNP and SNAP have been proposed to activate the latent, cytoplasmic form of NF-κB family proteins and allow their nuclear translocation (8) in a process which was suggested to involve the direct activation of guanine nucleotide-binding proteins by NO (34). Whereas more recent studies with human and bovine vascular endothelial cells have suggested that NO-donor treatment could inhibit NF-κB-dependent gene transcription (9), this inhibition of NF-κB activation being shown to result from the protection of IκBα from proteolytic degradation, and from the transcriptional upregulation of the IκBα gene (10). Further recent studies have shown that the NOS inhibitor NMMA can significantly enhance the activation of NF-κB DNA binding activity following treatment of a mouse macrophage cell line with bacterial lipopolysaccharide (LPS). The authors suggested that endogenous NO could have a negative feedback or modulatory role on the regulation of the NF-κB factor, possibly involving modification of the conserved NF-κB p50 cysteine 62 residue (35).

It was recently demonstrated that the promoter/enhancer region of the murine iNOS gene contains two κB motif binding sites for NF-κB family proteins, one of whose function is critical for the induction of iNOS gene expression in LPS-treated macrophages (25), similarly the human iNOS gene has a κB motif among the cytokine-responsive elements in its 5′ flanking region (26). These κB motifs would be consistent with studies on human brain microglial cells showing that LPS plus tumour necrosis factor-α

induction of iNOS gene expression could be inhibited by pre-treating cells with either SNP or NO gas solution (36). This presence of functionally important κB motifs in iNOS genes has interesting parallels with the transcriptional regulation of genes encoding IκB inhibitor proteins which normally function to maintain NF-κB family proteins in the cytoplasm and to inhibit their DNA binding activity. Thus, IκBα gene promoters have functionally important κB motifs (37–39), as have the NF-κB1 (p50 precursor) and NF-κB2 (p52 precursor) gene promoters (40,41).

Early studies of NF-κB-dependent HIV transcriptional enhancer activity indicated that chronic HIV infection of a human monocytic cell line resulted in increased NF-κB DNA-binding activity and activation of HIV proviral transcription (42). Recently, significant levels of iNOS mRNA were shown to be present 6–7 days after infection of normal human monocytes with HIV but to be undetectable in uninfected monocyte cultures (43). This correlated with NO levels, assayed as total nitrite formation, in the culture medium rising from being undetectable to 2–5 μM. Interestingly while HIV reverse transcriptase activity in the infected monocyte culture reached a maximum after 6 days, activity fell dramatically after this, meanwhile NO levels peaked sharply then declined after 7 days post-infection. It seems possible that this behaviour in HIV infected monocytes might have a periodic nature as an earlier study showed some evidence for long-term oscillations in reverse transcriptase activity in an HIV infected monocytic cell line (42).

Thus one scheme could envisage cell activation leading to the activation of DNA binding and the nuclear translocation of NF-κB—in turn causing the transcriptional activation of a range of genes (including the iNOS, IκBα and several NF-κB family genes). NO produced by newly expressed iNOS might then act in concert with newly synthesised IκB to inhibit the DNA binding activity of NF-κB and shut off the transcription of NF-κB-responsive genes to allow the cellular NF-κB/IκB system to return to its initial state.

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