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TRANSCRIPTIONAL INHIBITION OF THE INDUCIBLE NITRIC OXIDE SYNTHASE GENE BY COMPETITIVE BINDING OF NF-κB/REL PROTEINS

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The activity of the inducible nitric oxide synthase enzyme (iNOS) is tightly controlled, partly at the transcriptional level. We find NF-κB/Rel activation (p50-p50 and p50-p65) in RAW 264.7 macrophages after lipopolysaccharide treatment and binding to both NF-κB sites in the mouse iNOS promoter. To delineate the importance of NF-κB/Rel in iNOS gene transcription, we used an unusually direct approach to try to improve on the antioxidant-treatment or reporter techniques, namely the depletion of NF-κB/Rel activity through the use of a phosphorothioate-modified oligonucleotide containing three copies of the NF-κB consensus sequence. The reduction in NF-κB/Rel activity (particularly that binding to the downstream of the two sites) was associated with a 50% reduction in NO output and a reduction in the quantity of the iNOS protein expressed. These results point to the probability that physiologically relevant NF-κB/Rel activators or repressors other than lipopolysaccharide might crucially affect the macrophage NO response.

The recent explosion of scientific interest in the seemingly endless number of normal and aberrant processes involving nitric oxide (NO) has naturally been accompanied by investigations into the means by which a cell controls its NO production. Because of the pathological effects of NO on DNA synthesis and mitochondrial respiration (1,2), it was to be expected that the enzymes

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Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa B; LPS, lipopolysaccharide; PS, phosphorothioate; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; U, upstream putative κB site; D, downstream putative κB site; mut κB, phosphorothioate-modified oligomer containing three mutated copies of the κB consensus sequence; κB, phosphorothioate-modified oligomer containing three copies of the κB consensus sequence.

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responsible for the generation of NO from the oxidation of L-arginine, would be tightly controlled. Until now, two constitutive and one inducible nitric oxide synthase (iNOS) genes have been described in humans (3-5). The inducible enzyme is independent of intracellular rises in the Ca^{2+} concentrations unlike the constitutive isoforms (6). Instead, iNOS is believed to be controlled at several stages in its synthesis (7), and there is evidence for transcriptional activation of the iNOS gene from studies using the mouse macrophage cell line RAW 264.7 (7,8). In these studies, a 5' flanking region of the mouse iNOS gene was sequenced, many putative *cis*-acting regulatory elements were identified, and the relative importance of different parts of the whole region was described using different reporter constructs. Both upstream and downstream portions of the control region 5' to the start of transcription were found to contain a putative NF-κB binding site (8,9), and moreover, activation of members of the NF-κB/Rel transcription factor family by lipopolysaccharide (LPS) has been well documented (10). Nevertheless, although there is a broad consensus that the upstream part of the 5' flanking region (-913 to -1029) is necessary for stimulation of iNOS transcription by interferon-γ, and a downstream part (-48 to -201) is necessary for stimulation by LPS, one group has found the upstream part was necessary for a full LPS-mediated iNOS induction (9), while another did not (8). Recent work using a reporter construct of the downstream portion of the control region has pointed to the importance of NF-κB/Rel proteins in iNOS induction (11). However, reporter assays can be criticised in that the transfected DNA might not adapt a configuration identical to the native DNA (9), and in any case they do not take into account the chromatin structure in the region of the promoter of interest. Other studies have linked rather more indirectly the NF-κB/Rel proteins to iNOS induction, by the use of an antioxidant, pyrrolidine dithiocarbamate, said to have specific inhibitory effects on NF-κB/Rel activity (12,13). However, we have found a macrophage toxicity associated with this compound that is too great to justify its role as a specific inhibitor (unpublished observations, C.G and J-F J).

To learn more about the means by which macrophages control their output of NO, and the role of NF-κB/Rel in this, we have attempted to use a fairly novel approach, one which has only been rarely described before, for example by Eck *et al.* to define the importance of NF-κB in the activation of expression of leukocyte integrin CD11b (14). So, as well as identifying the proteins binding to the upstream as well as the downstream NF-κB sequences in the iNOS promoter, we have used a phosphorothioate-modified double-stranded oligonucleotide containing three copies of the NF-κB consensus sequence as a means of diminishing the activity of NF-κB/Rel proteins in macrophages. Using such a transcription factor "trap" makes possible a direct analysis of the effects of a *trans*-acting regulatory protein on the expression of integral intracellular genes, and it has given us direct evidence for the role of NF-κB/Rel proteins in the control of iNOS transcription.

Materials and Methods

Phosphorothioate oligonucleotides - The phosphorothioate (PS) oligomers were synthesized and purified as described elsewhere (15,16). The κB sequence is (with the consensus binding site

underlined) GGGGACTTTCCGCTGGGGACTTTCCAGGGGGACTTTCC; the mutated κB sequence is GTCTACTTTCCGCTGTCTACTTTCCACGGTCTACTTTCC. The sequences were annealed to their complementary strands, *in vitro*, by mixing molar equivalents in 1X annealing buffer (20 mM Tris.HCl, pH 7.5, 20 mM $MgCl_2$ and 50 mM NaCl) to give the κB and mut κB oligomers. The mixture was heated to 100°C for 12 min and allowed to reach room temperature slowly, over two hours. The annealed oligomers were stored at -20°C.

Oligonucleotide probes - Single-stranded oligonucleotides were synthesized on a Millipore Cyclone Plus automated synthesizer, and purified on an oligo-pak cartridge (Millipore S.A., Yvelines, France). Complementary strands were annealed to form oligomers U and D (as shown below), which correspond to the upstream (U) and downstream (D) putative NF-κB binding areas from the 5' flanking region of the mouse iNOS gene; the sites believed to be involved in binding are underlined.

-975	-959	-89	-73
TAGGGGGATTTTCCCTC		ACTGGGGACTCTCCCTT	
ATCCCCCTAAAAGGGAG		TGACCCCTGAGAGGGAA	
Oligomer U		Oligomer D	

Oligomers U and D were end-labelled with (γ-^{32}P)ATP, obtained from Du Pont de Nemours, Les Ulis, France, using T4 polynucleotide kinase, from Pharmacia, Yvelines, France, to give probes U and D.

Reagents - LPS (from E. coli serotype 0128:B12) and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Sigma. Poly (dI-dC)·poly (dI-dC) was from Pharmacia, and the supershift antibodies were from Santa Cruz Biotechnology, Tebu, Le Perray en Yvelines, France.

Cell culture - RAW 264.7 cells (an established mouse macrophage cell line) were a kind gift of Dr S.B. Corradin, Institute of Biochemistry, Epalinges, Switzerland, and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (endotoxin-free), and gentamycin (40μg/ml). The cells were verified free of mycoplasma contamination.

Nitrite assays - NO_2^- concentrations in the culture media were determined using the Griess assay (17). The concentrations are representative of the amount of NO produced by cells (18).

Gel shift assays - Nuclear protein extractions were carried out as described (19). Extracts (4 μg) were incubated in the presence or absence of cold competitor or non-competitor (NF-κB binding site of the IL-2Rα promoter, or a mutated version of the same sequence, respectively) for 10 min, in an incubation medium of (final concentrations) 20 mM HEPES pH 7.5, 35 mM NaCl, 2 mM DTT, 60 mM KCl, 0.01% NP-40, 0.1 mg/ml BSA and 0.2 mg poly (dI-dC)·poly (dI-dC). Probe U or D (approx 50,000 cpm) was then added and after 20 mins at room temperature, the reaction mixtures were deposited in the wells of 5% polyacrylamide gels under a low voltage (25 V), and the products were separated by electrophoresis at 160 V for 2 h. Anti- NF-κB/Rel subunit antibodies (1 μg) were either added just after adding the protein extracts and incubated at 4°C overnight (as here), or added after the probe and incubated as normal. Similar results were obtained in either case.The gels were fixed in acetic acid/methanol/water (10:10:80), dried, and analysed by autoradiography.

Western analysis - Cytosolic fractions were prepared from the same cell preparations used for nuclear protein extraction, and stored at -80°C after the addition of glycerol (20% final concentration). Equal quantities of total cytosolic protein (13 μg) were then separated by SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was then incubated with a 1:1000 dilution of polyclonal rabbit anti-rat iNOS antiserum (which cross-reacts with the mouse iNOS). The membrane was washed and then probed with horseradish peroxidase-conjugated goat anti-rabbit IgG at a dilution of 1:4000. Detection of the iNOS protein was performed using the Amersham ECL detection kit according to the manufacturer's instructions.

Results and Discussion

NF-κB/Rel proteins bind to both putative binding sites in the murine iNOS promoter - We began by looking at the NF-κB/Rel proteins activated by LPS which might bind to their putative binding sites U (for upstream) and D (for downstream), *in vitro*. We found at least two proteins binding to U and D after LPS treatment (100 ng/ml, 3 h), and at least one which bound with or without treatment (Fig. 1A). The two proteins activated by LPS were found to be specifically binding to the U and D NF-κB sites, whereas the other protein was non-specific (Fig. 1B). The proteins binding specifically to probe U were identified as probably consisting of a p50-p50 homodimer (band 1) and a p50-p65 heterodimer (band 2) (Fig. 1C). Identical results were also obtained using probe D (not shown). The presence of c-Rel binding to the downstream site has already been described (11) and the continued presence of some of band 2 even after the addition of both anti-p50 plus anti-p65 antibodies to LPS-stimulated nuclear proteins (result not shown) lends credence to its binding here, although we did not test this. It should be stressed that the shifts we observed were similar using either probe, and that they appeared fairly different to those obtained in an earlier study on RAW 264.7 cells using either a long or a short downstream NF-κB probe from the murine iNOS promoter (11). The fundamental differences were that we found less p50 homodimer in the nucleus prior to LPS activation, and that a non-specific binding protein was always present whichever probe was used. We found no binding of RelB or p52 subunits to either of the iNOS NF-κB sites.

Direct inhibition of NF-κB/Rel using a transcription factor trap diminishes iNOS activity - We used a PS-modified consensus NF-κB sequence-containing oligonucleotide (κB) and a mutated version

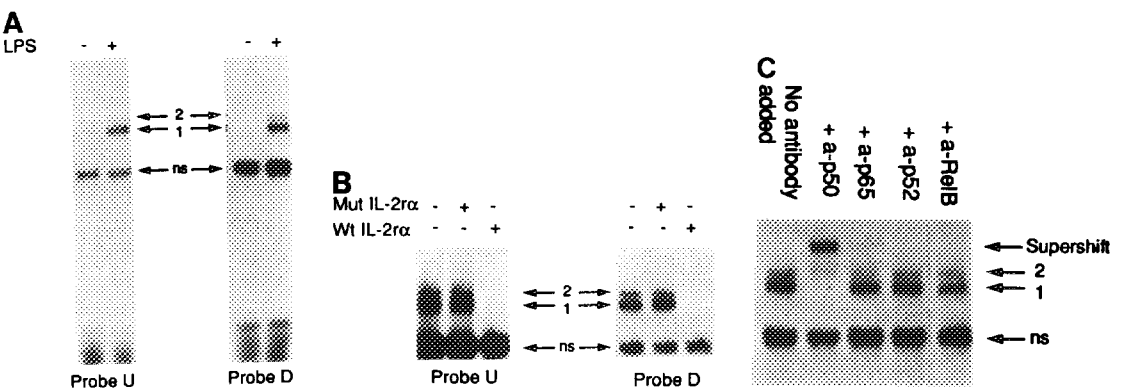

Fig. 1. NF-κB proteins bind to both putative binding sites in the mouse iNOS promoter. RAW 264.7 cells grown in 75-cm^2 flasks were treated with 100 ng/ml LPS or without for 3 h prior to extraction of nuclear proteins and gel shift analysis using probes U and D. (A) Assay of proteins binding to probes U and D. (B) Specificity of binding. Binding of LPS-activated NF-κB/Rel proteins was carried out in the presence (+) or absence (-) of a 30-fold excess of a competitor (Wt IL-2Rα) or non-competitor (Mut IL-2Rα). (C) Identification of U-binding proteins using anti-p50, anti-p65, anti-p52, or anti-Rel B. ns: non-specific band.

of the same sequence (mut κB) as a control. In earlier experiments on RAW 264.7 cells, the pattern of NF-κB/Rel proteins binding to the NF-κB consensus sequence was similar to those binding the U and D probes (result not shown). Neither κB nor mut κB interfered with the Griess assay. At initial concentrations of 20 μM (and 10 μM during LPS treatment for 24 h), we saw a large difference in NO production between the κB- and the mut κB-treated cells, without a large effect on viability (Table 1). The reduction in NO production was also confirmed as being due to a diminution in the quantity of iNOS protein present in the κB-treated cells (Fig. 2). It should however be noted that treatment with the mut κB control diminished NO production by 50% and caused a similar decrease in the quantity of the iNOS protein (Fig. 2), compared to a PBS-treated sample. Nevertheless, as has been greatly debated for the non-specific effects often found in the use of PS-modified oligonucleotides in general as inhibitors of gene expression, there is a clear necessity that mutated oligonucleotides are used as controls so as to judge the specificity of antisense or trap molecules (for reviews see 20 & 21). Many of these background PS effects might be attributable to their recently described action in specifically activating the Sp-1 transcription factor (22). So in effect, it is not surprising that we found a non-NF-κB-specific effect of the mut κB control compared to the PBS-treated cells. Of course, we could also have tested other variations of PS-modified oligonucleotide sequences as controls, but we felt that this was not justified since the number of possible sequences to use was virtually limitless.

We then confirmed that in the κB-treated cells, binding of p50-p50 and p50-p65 to both the U and D sequences in the mouse iNOS promoter was greatly diminished (Fig. 3). This was not seen in either the mut κB-treated or the PBS control cells, which demonstrates the non-NF-κB-dependent background effect of mut κB on NO production. It was interesting to see that the reduction in binding was more for the D than the U sequence. It is tempting to speculate that this fits with the results of the reporter assays that indicate that the downstream site is probably more important than the upstream one in the macrophage iNOS response to LPS (9, 11). That we did not see a greater

Table 1. Inhibition of NO production in oligonucleotide-treated cells. RAW 264.7 cells were seeded in 96-well plates at 2 x 10^5/well and treated with 60 μl of medium containing 20 μM of mut κB, 20 μM of κB, or a volume of PBS equivalent to that containing the concentrated oligonucleotides for 4 h. 60 μl of LPS-containing medium (200 ng/ml) was then added to each of the wells, and the cells were incubated for 24 h (i.e.,the concentrations over the final 24 h were 10 μM of PS-oligonucleotide and 100 ng/ml LPS) prior to taking of samples of media (100μl) for nitrite analysis. Cell viability was measured as a percentage of the untreated cells using the MTT test (28). Data given are means ± standard error (n=8). The results shown here are representative of three separate experiments, carried out on separate occasions.

Treatment	Nitrite (nmoles released into 100μl of cell medium)	% Cell viability compared to PBS-treated cells
Mut κB control	0.08 ± 0.01	117 ± 9.2
κB	0.04 ± <0.01	89 ± 9.8

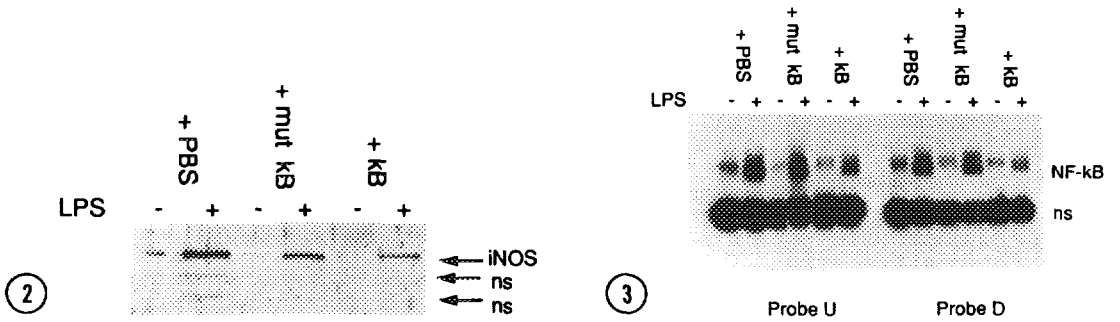

Fig. 2. Inhibition of NF-κB/Rel activity in κB-treated cells. RAW 264.7 cells were grown in 60 x 15-mm dishes and treated with 2 ml of medium containing 20 μM of mut κB, 20 μM of κB, or a volume of PBS equivalent to that containing the concentrated oligonucleotides for 4 h. Medium (2 ml) with LPS (200 ng/ml) or without was then added to each of the dishes, and the cells were incubated for 16 h (i.e.,the concentrations over the final 16 h were 10 μM of PS-oligonucleotide and 100 ng/ml LPS) prior to several cell washings, nuclear protein extraction, and gel shift analysis.

Fig. 3. Western analysis of iNOS in PS-modified oligonucleotide-treated cells. RAW 264.7 cells were grown in 60 x 15-mm dishes, and treated with 2 ml of medium containing 20 μM of mut κB, 20 μM of κB, or a volume of PBS equivalent to that containing the concentrated oligonucleotides for 4 h. Medium (2 ml) with LPS (200 ng/ml) or without was then added to each of the dishes, and the cells were incubated for 16 h (i.e.,the concentrations over the final 16 h were 10 μM of PS-oligonucleotide and 100 ng/ml LPS) prior to several cell washings, cytosolic fraction preparation, and Western analysis. The strongly staining band at 130 kdal. is presumed to be iNOS (molecular weight markers were also run - not shown).

inhibition of iNOS synthesis by the κB oligonucleotide was not surprising, since it is unlikely that the expression of a gene as crucial as iNOS would be transcriptionally activated by only one factor - especially since there is such a plethora of potential transcription factor binding sites in the murine iNOS promoter (8, 9). Indeed the latest evidence points to the involvement of other proteins that might interact with NF-κB/Rel proteins in the transcriptional activation process (11), as well as the importance in mouse primary macrophages and RAW 264.7 cells of the IRF-1 transcription factor (23, 24). Nevertheless, the participation of NF-κB/Rel in the macrophage NO response is important, because of the plethora of NF-κB/Rel regulating agents such as protease inhibitors (25), oxygen radicals or antioxidants (26), or even salicylates (27), whose actions might now at least partially be shown to be via NO.

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