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Non-NF-κB elements are required for full induction of the rat type II nitric oxide synthase in vascular smooth muscle cells

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1 We have investigated the role of the NF-κB binding sites and other promoter elements beyond NF-κB in iNOS induction in rat vascular smooth muscle cells (SMC).

2 Rat aortic SMC transfected with iNOS promoter constructs with either mutation or deletion of the downstream NF-κB site exhibited about 50% reduction in promoter activity in response to a cytokine mixture, whereas either mutation or deletion of the upstream NF-κB site reduced promoter activity by 90%, suggesting that the latter site is the most important, and that co-existence of two NF-κB sites is necessary for iNOS induction.

3 Nuclear NF-κB activity was robustly induced by TNF-α. However, TNF-α alone did not induce iNOS promoter activity, protein expression, or nitrite production, indicating that NF-κB activation alone is not sufficient for iNOS induction.

4 The construct up to −890 bp, containing the downstream NF-κB site, exhibited little response to cytokines. The construct up to −1.0 kb, containing the two NF-κB sites exhibited only 22% of full promoter activity. The regions −1001 to −1368 bp and −2 to −2.5 kb contributed an additional 43 and 22% promoter activity, respectively.

5 Internal deletion or reversal of the orientation of −1001 to −1368 bp in the full promoter resulted in 40% reduction in promoter activity.

6 These data suggest that the co-existence of two NF-κB sites is essential for core promoter activity, but that full induction of the rat SMC iNOS gene requires other elements located between −1.0 to −1.37 and −2.0 to −2.5 kb of the promoter.
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Abbreviations: CM, cytokine mixture containing IFN-γ, TNF-α and IL-1β; iNOS, inducible nitric oxide synthase; MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NO, nitric oxide; RASMC, rat aortic smooth muscle cells

Introduction

Nitric oxide (NO) plays important roles in the physiology and pathophysiology of many organ systems (Moncada *et al*., 1991; Nathan & Xie, 1994). Generation of NO from its substrate, L-arginine is catalyzed by NO synthase (NOS) (Stuehr, 1999). Of the three isozymes of NOS, two are constitutively expressed primarily in vascular endothelia cells (eNOS or type III) and neuronal cells and skeletal muscle (nNOS or type I). A third type, the inducible NOS (iNOS or type II), is induced after immunologic or inflammatory stimuli, such as cytokines or gram negative bacteria. The induction of iNOS produces high output of NO (Hecker *et al*., 1999), and has been proposed as a major factor involved in the pathologic vasodilatation and tissue damage observed in patients with, and animal models of, sepsis and septic shock (Kilbourn *et al*., 1990; Titheradge, 1999; Wright *et al*., 1992), as well as for the side-effects of anti-tumour therapy with cytokines (Hibbs *et al*., 1992). Understanding the molecular mechanisms of iNOS induction will provide the fundamental basis for developing agents to control gene activation in pathological conditions.

More and more data indicate that there are species-dependent variations in the molecular mechanisms of iNOS gene induction, and it appears that the higher the species, the more complex and longer the iNOS gene promoter, suggesting an evolutionary change in the mechanisms of iNOS induction. The avian iNOS gene promoter has been sequenced, and its regulatory region is located exclusively within the first 300 bp upstream of the transcriptional starting site of the gene (Lin *et al*., 1996). Promoter analysis of the mouse iNOS gene revealed that about 1 kb of the 5′-flanking region from the transcription starting site confers full promoter activity in response to cytokines and LPS (Kim *et al*., 1997; Spink *et al*., 1995; Xie *et al*., 1993). The murine iNOS promoter includes two regulatory regions, region I (RI) in position −48 to −209 bp and region II (RII) in position −913 to −1029 bp (Lowenstein *et al*., 1993; Xie *et al*., 1993). The rat and murine iNOS promoters exhibit 73% sequence homology. However, for the full induction of the rat iNOS promoter, an additional 2 kb of the 5′-flaking region is necessary (Zhang *et al*., 1998). In man, a −1.09 kb of the 5′-flanking region of the iNOS gene does not respond to LPS (Kleinert *et al*., 1996) and the first −3.8 kb upstream of the transcription starting site results in only basal promoter activity, demonstrating at most a slight response to cytokines (De Vera *et al*., 1996; Spitsin *et al*., 1996). A 3–5 fold induction is found in promoter segments containing up to −5.8 and −7.0 kb, and a 10 fold activation is observed in constructs containing −16 kb of the 5′-flanking region of the gene (De Vera *et al*., 1996).

The mechanisms of transcriptional regulation of iNOS induction have been predominantly characterized in the mouse macrophage cell line, RAW 264.7 (Lowenstein *et al*., 1993; Xie

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& Nathan, 1994; Xie *et al*., 1993); a number of transcriptional binding sites have been identified. An octamer (Oct) binding site (in position −54 to −61 bp) may be important in mediating the induction of iNOS (Gay *et al*., 1998; Xie, 1997) by binding the high mobility group-I (Y) protein which facilitates NF-κB binding (Pellacani *et al*., 1999; Perrella *et al*., 1999). The mutation or deletion of the Oct binding site significantly reduces the inducibility of iNOS (Perrella *et al*., 1999; Xie, 1997). The synergistic effect of interferon-γ with LPS on iNOS induction is dependent on the RII since IRF-1 induced by IFN-γ binds to the IRF-E in position −923 to −913 bp (Martin *et al*., 1994; Spink & Evans, 1997). The importance of other transcriptional binding sites, such as GAS in position −942 bp of the mouse promoter (Gao *et al*., 1997) and AP-1 in position −5301 and −5115 bp of the human promoter (Marks-Konczalik *et al*., 1998), in mediating iNOS induction has also been reported. However, the transcriptional binding sites mostly responsible for cytokine- or LPS-mediated iNOS induction are the NF-κB binding sites. RI contains a 'downstream' NF-κB binding site (in position −76 to −85 bp) and functions as the core promoter since a construct of −1 to −85 bp confers remarkable induction in RAW 264.7 cells (Kim *et al*., 1997; Xie *et al*., 1994). A second, 'upstream' NF-κB binding site (in position −971 to −962 bp) located in RII may be required for maximal induction of iNOS (Kim *et al*., 1997).

In rats administered LPS, vascular smooth muscles cells (SMC) represent the predominant cell type expressing iNOS (Liu *et al*., 1997) suggesting that NO derived from vascular SMC may account for the hyporeactivity of the vessel wall to inotropes which is associated with septic shock. Thus, the signal transduction pathways and the molecular regulation of iNOS induction in vascular SMC are potentially important therapeutic targets. Few studies have focused on the molecular mechanisms of iNOS induction in vascular SMC. Spink and colleagues have transfected the murine iNOS promoter in a rat SMC line, A7r5, to study the role of the NF-κB/Rel family in iNOS gene activation (Spink *et al*., 1995). By deletion and mutagenesis analysis, the upstream NF-κB site, as well as a number of interferon response elements, have been identified as major elements in response to cytokine stimulation. The residual promoter activity was attributed to the independently functioning downstream NF-κB site (Spink *et al*., 1995). In contrast, Perrella and co-workers have shown that the construct containing −234 to +31 bp (with a downstream NF-κB site) of the murine iNOS promoter confers most of promoter activity in response to IL-1β stimulation when transfected into primary cultured rat aortic SMC (Perrella *et al*., 1996b). Recently, we have cloned various lengths of the 5′-flanking region of the rat iNOS gene (Zhang *et al*., 1998). The sequence of the downstream NF-κB binding site is well conserved between rat and mouse, but the induction of the rat iNOS in vascular SMC is remarkably different from that in the mouse. For example, a −484 bp rat iNOS promoter DNA construct which contains the downstream NF-κB binding site does not respond to cytokines or LPS in the rat SMC, and full promoter activity is observed at and beyond the −3.2 kb construct which includes an upstream NF-κB binding site (Zhang *et al*., 1998). Thus, it remains controversial which NF-κB site is the most important in iNOS induction in vascular smooth muscle cells.

Recent studies indicate that other promoter elements may play important roles in iNOS gene induction, even though NF-κB binding sites, especially the downstream one, as well as Oct, IRF-E, and GAS, appear to be crucial in murine macrophages. TGF-β suppresses iNOS mRNA and protein expression both *in vitro* and *in vivo* (Perrella *et al*., 1994; 1996a), but TGF-β does not affect nuclear NF-κB binding activity, suggesting element(s) independent of NF-κB in iNOS induction (Perrella *et al*., 1996a). The same observation has been reported with endothelin-1 mediated inhibition of iNOS induction (Beck & Sterzel, 1996); moreover, iNOS can be induced in the absence of active NF-κB in rat mesangial cells (Nakashima *et al*., 1999). An iNOS gene-deficient mouse has been generated with an targeted deletion of the proximal 585 bp of the promoter (including RI), as well as exon 1–4. However, the generation of a cerebral infarct in these mice initiates transcriptional-mediated expression of the disrupted iNOS gene even in the absence of RI (Loihl *et al*., 1999). Together, these data suggest that non-NF-κB binding sites may be necessary for maximal iNOS gene induction.

The present study was undertaken to investigate whether NF-κB binding sites are essential in iNOS gene induction in rat SMC, and whether other potential elements beyond NF-κB may be responsible for the full induction of iNOS gene in rat vascular smooth muscle cells. In the past, similar studies have utilized heterologous systems (murine promoter transfected into rat cells). In consideration of the potential differences in iNOS activation among different species, we have employed a homologous system (rat iNOS promoter transfected into rat SMC) to eliminate possible species differences in the regulation of iNOS. Furthermore, we employed fresh cultures of SMC, sub-cultured no more than four times, since it has been reported that the SMC cell line A7r5 does not possess the p50 subunit, which, together with p65, forms NF-κB (Spink *et al*., 1995).

Methods

Reagents

Recombinant human IL-1β was obtained from Boehringer (Indianapolis, IN, U.S.A.). TNF-α and rat IFN-γ were from R & D Systems (Minneopolis, MN, U.S.A.). Tissue culture media and Lipofectamine were obtained from GIBCO/BRL (Grand Island, NY, U.S.A.), and foetal bovine serum (FBS) was purchased from HyClone (Logan, UT, U.S.A.).

Cell culture

Rat aortic smooth muscle cells (RASMC) were harvested from Wistar rats (Harlan) by enzymatic dissociation using standard methods (Geisterfer *et al*., 1988). The cells were identified as smooth muscle by indirect immunofluorescent staining for α-actin, using mouse anti-α-actin antibody and anti-mouse IgG FITC conjugate. The positive stain was routinely 100%. RASM cells were grown in 100-mm plates in 50% F12 nutrient medium and 50% Dulbecco's Modified Eagle Medium supplemented with 10% FBS, glutamine, penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}) and were sub-cultured (1 : 4) into 100-mm tissue culture plates. Cells at passage 3 or 4 were used in the studies, obtained from 17 separate harvests. Each harvest contained cells pooled from four rats. All cultures were grown in a humidified incubator at 37°C under 5% CO_2 in air.

Generation of mutation and deletion constructs

We have recently cloned the 3281 bp 5′-flanking region of the rat iNOS gene consisting of upstream −3196 bp and downstream +85 bp from the transcription starting site (Zhang *et al*., 1998). The 5′-end was linked to a 25-bp adapter with *Mlu*I,

*Sal*I and *Sma*I sites. The 3281 bp segment was cloned into *Mlu*I-*Xho*I sites upstream of the luciferase gene in a promoterless pGL3-Basic vector (Promega Madison, WI, U.S.A.). The −3.2 kb construct conferred full promoter activity in response to LPS or a cytokine mixture since a similarly cloned −5.1 kb construct of the rat iNOS promoter showed no further increase in inducibility to the same stimuli (Zhang *et al*., 1998). Upstream and downstream NF-κB mutants and deletions were generated by site-directed mutagenesis using the single stranded DNA method (Zhang *et al*., 1996). In order to identify the minimal length of the fully active promoter, a series of deletion constructs were obtained by digesting the −3.2 kb rat iNOS promoter with unique restriction enzymes and re-ligating the linearized plasmid DNA: −135 bp construct with *Nru*I; −1002 bp construct with *Nco*I; −1368 bp construct with *Sph*I; −2051 bp construct with *BspE*I; and −2493 bp construct with *BstE*II. PCR-generated deletion constructs were created with a reverse primer at the 3′-end of the promoter and the following forward primers: −893 bp construct, 5′-CCATGCCATGTATGAATCGTTGTAGG-3′ (−893 to −868 bp); −1000 bp construct, 5′-GGGATGATGAGTGGACCCTGGCGGG-3′ (−1000 to −975 bp); −1709 bp construct, 5′-GTCGACTTTGATATACTGAAATTC-3′ (−1709 to −1686 bp) with incorporation of a *Mlu*I or *Sma*I site at the 5′-end. The 3.2 kb segment was the template DNA, and the PCR products were digested with *Mlu*I or *Sma*I and *Xho*I and ligated into the pGL3-Basic vector. Using a forward primer at upstream of −3.2 kb and a reverse primer, 5′-CATGCATGCATGCACACACACGCCCCCTCC-3′ (−1398 to −1369 bp), a PCR product (−3196 to −1369 bp) was obtained. The PCR product was ligated to the upstream of −1000 bp deletion construct with blunt ends to create an internal deletion of the fragment between −1001 and −1368 bp. In all cases, the identity of the DNA constructs was confirmed by sequencing.

Transient transfection of DNA into RASMC and the luciferase activity assay

Transfection with Lipofectamine or Lipofectamine Plus was used according to published procedures (Zhang *et al*., 1996). Briefly, plasmid DNA with equal molar amounts of each DNA construct were mixed with serum-free culture medium to a final volume of 400 μl. To keep the total amount of DNA (2 μg) constant, carrier DNA pGL3-Basic was added with the smaller constructs. The DNA mixture was gently added to an equal volume of diluted Lipofectamine (22 μl Lipofectamine + 378 μl serum-free medium) and incubated at room temperature for 30 min., whereupon a further 3.2 ml of medium was added. This mixture was added in 0.68 ml aliquots to each well of RASMC (80% confluent) in 12-well plates, these cells being previously washed with serum-free medium. After incubating at 37°C for 3–5 h, 0.17 ml medium containing 50% FBS was added to the cells (final concentration of FBS 10%), and the incubation continued for another 8–10 h. The transfected cells were then changed to fresh medium. Antibiotics were not present in the transfection mixture during the transfection period. To control for the efficiency of transfections between different constructs, a plasmid DNA containing a cytomegalovirus promoter driven β-galactosidase gene was co-transfected. The transfected cells were incubated with TNF-α (150 u ml^{-1}), or IFN-γ (150 u ml^{-1}), or IL-1β (250 u ml^{-1}) or a mixture of the three cytokines (CM) for the indicated time. These concentrations are similar to those used by other investigators in vascular SMC (Perrella *et al*., 1996b; Spink *et al*., 1995). After being washed three times with PBS, cells were lysed with 0.35 ml of 1 × Cell Culture Lysis Reagent, and the luciferase activity in 20 μl of cell lysate was measured with the Luciferase Assay Substrate (Promega) in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, U.S.A.).

Western blotting analysis of iNOS protein

At the end of experimental treatments, cells (in 6-well plates) were washed three times with ice-cold PBS. Then, 0.35 ml ice-cold RIPA lysis buffer (20 mM Tris-HCl pH 7.4, 2.5 mM EDTA, 1% Triton, 10% glycerol, 0.1% SDS, 10 mM Na_4P_27, 1 mM PmsF and 100 mM NaF) was added. The lysates containing equal amounts of protein were subsequently loaded on 7.5% SDS polyacrylamide gels, and the resolved proteins were electrophoretically transferred to a nitrocellulose membrane. The nonspecific binding to the membrane was blocked by 5% nonfat dry milk in PBS. iNOS protein was detected by specific polyclonal mouse anti-iNOS antibody with 1 : 5000 dilution (Transduction Laboratories, Lexington, KY, U.S.A.). The second antibody was a peroxidase-conjugated goat anti-mouse IgG. Membrane was developed with the enhanced chemiluminesce detection system (Amersham, Piscataway, NJ, U.S.A.) and exposed on film.

Nuclear extracts

Confluent RASMC were treated with an individual cytokine or CM for 30 min. Nuclear proteins were isolated with the modified protocol by Dignam *et al*. (1983) at 4°C. Cells were washed with ice-cold PBS, collected by gently scraping off the plates using a cell lifter, followed by centrifugation. Cells were re-suspended in five volumes of hypotonic buffer (in mM): HEPES-KOH (pH 7.9) 10, KCl 10, $MgCl_2$ 1.5, DTT 0.5, PMSF 0.5 for 15 min on ice, and homogenized by passing 10 times through a 27-gauge needle in the presence of 0.5% NP-40. After centrifugation at 3300 × *g* at 4°C for 15 min and one wash with hypotonic buffer, the pellet was re-suspended in salt buffer (in mM): HEPES-KOH (pH 7.9) 20, KCl 400, $MgCl_2$ 1.5, EDTA 0.2, 25% glycerol, DTT 0.5 and PMSF 0.5. The re-suspended nuclei solution was stirred using a rotator for 30 min at 4°C, and the supernatants were collected by centrifugation at 20,000 × *g* at 4°C for 30 min.

Electrophoretic mobility shift assay (EMSA)

The NF-κB oligonucleotide was derived from the rat iNOS promoter (−972 to −949) containing the up-stream NF-κB binding site (underlined): 5′-TGCCAGGGGGATTTTCCCCTCT-3′, and 5′-GAGAGAGGGAAAATCCCCCTGG-3′. Each oligomer was filled with [α-^{32}P]-dCTP and the three other non-radiolabelled dNTPs by the Klenow fragment of DNA polymerase I. Nuclear protein (5 μg) was incubated with 340,000 c.p.m. of ^{32}P-labelled oligonucleotide at 30°C for 30 min in the gel shift binding buffer (in mM): HEPES 12, 10% glycerol, Tris-HCl 4, KCl 60, EDTA 1, DTT 1, 2 μg poly(dI-dC), and 2.5 μg BSA, in a final volume of 15–20 μl. Subsequently, the free and the oligonucleotide-bound protein were separated by electrophoresis on a native 5.5% polyacrylamide gel in 0.5 × Tris borate-EDTA buffer. After electrophoresis, the gel was dried and exposed to Hyperfilm™ MP. The intensity of the bands was analysed with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Competition experiments were conducted by adding excess unlabelled NF-κB oligonucleotide in the binding reaction mixture.

Nitrite assay

Nitrite in RASM cell culture supernatants was measured spectrophotometrically using the Griess reagent (Green *et al*., 1982).

MTT assay

The cytotoxicity of cultured RASMC in the presence of different cytokines was analysed by the MTT method (Carmichael *et al*., 1987). MTT is reduced by dehydrogenase enzymes of viable cells to formazan, a purple crystal product. The colour development of formazan is obtained by adding solvent DMSO, and the OD is measured in a spectrophotometer.

Statistical analysis

Values are reported as means ± s.e.mean. Significant differences among means were estimated by ANOVA. Statistical significance was established at $P<0.05$.

Results

iNOS gene induction by cytokines in RASMC

Rat aortic SMC produced little amount of nitrite (Figure 1a), and there was no detectable iNOS protein (Figure 1c). Small basal luciferase activity was observed in RASMC transfected with the full promoter (3.2 kb construct), and this was similar to the activity from cells transfected with the pGL3-Basic vector (Zhang *et al*., 1998). When RASMC were stimulated with a mixture of TNF-α, IFN-γ and IL-1β (CM), robust increases in nitrite accumulation, luciferase activity, and iNOS protein expression were observed (Figure 1a–c). In order to determine the cytokine which could most effectively induce iNOS expression, RASMC were stimulated with TNF-α, IFN-γ, and IL-1β alone or in various combinations. The induction of iNOS promoter, protein and nitrite production in RASMC exhibited a consistent pattern in response to the different cytokines; the strongest induction was achieved with the combination of the three cytokines (CM) (Figure 1). IL-1 was the only single agent to significantly induce luciferase activity, iNOS protein and nitrite production, reaching about 40–50% of CM-induced levels in RASMC. Whereas, neither TNF-α nor IFN-γ alone, or the combination of both induced iNOS promoter activity, protein and nitrite production in SMC, either TNF-α or IFN-γ potentiated the iNOS-inducing effects of IL-1β (Figure 1). The cytotoxicity of different cytokines on RASMC was evaluated using the MTT procedure; Figure 1d shows that there was no cytotoxicity by the cytokines used in the study.

Role of NF-κB in the induction of iNOS in RASMC

NF-κB binding sites in the murine iNOS promoter play an important role in the induction of macrophage iNOS. To investigate the role of NF-κB binding sites in the induction of RASMC iNOS, we created 3.2 kb mutant promoter constructs

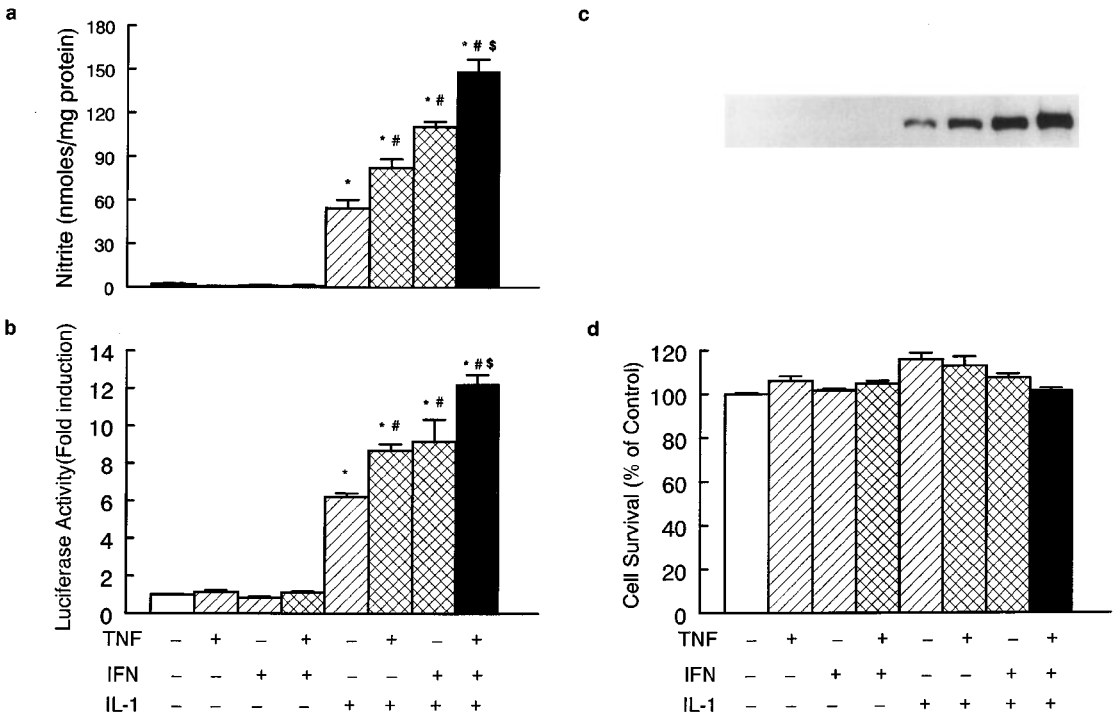

Figure 1 Induction of rat iNOS promoter activity, protein expression, nitric accumulation and cytotoxicity by cytokines in rat aortic SMC. (a) Confluent RASMC were incubated with individual cytokines or combinations for 24 h and nitrite production was determined by the Griess reaction. Data are presented as mean ± s.e.mean, $n=5-7$ wells per bar, from two separate experiments and two separate RASMC harvests. (b) The 3.2 kb rat iNOS promoter was transfected into RASMC, stimulated with individual cytokine or combinations for 6 h and then luciferase activity was analysed. Data are presented as mean ± s.e.mean, $n=3-4$ wells per bar from one experiment and one RASMC harvest. (c) Confluent RASMC were incubated with individual cytokine or combinations for 24 h and the iNOS protein expression was analysed by Western blot. Data represents three separate experiments with cells obtained from two different harvests. (d) Confluent RASMC were treated as in (a), and cells were incubated with MTT. The colour development was by addition of DMSO. The ODs from controls are expressed as 100%. Data are presented as mean ± s.e.mean, $n=6-8$ wells per bar from three separate experiments and two separate RASMC harvests. No significant difference among Control, TNF-α, IFN-γ and TNF-α + IFN-γ in luciferase activity and nitrite production; $*P<0.05$, compared to control; $\#P<0.05$, compared to IL-1β; and $\$P<0.05$, compared to IL-1β + TNF-α or IL-1β + IFN-γ by ANOVA.

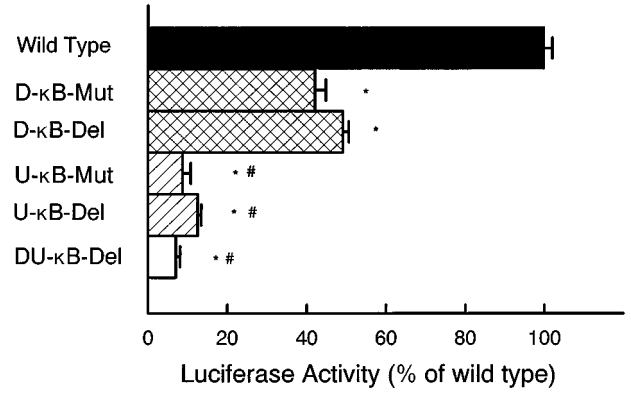

Figure 2 The effects of mutating or deleting the down-stream (D-κB-Mut. or Del.) and the up-stream NF-κB sites (U-κB-Mut. or Del.) on the induction of iNOS promoter in response to CM in RASMC. In the 3.2 kb iNOS promoter, downstream NF-κB or upstream NF-κB site was mutated (GGG to CTC) or deleted, and the construct DNA was transfected into RASMC, stimulated with CM for 6 h and then luciferase activity was analysed. Data are presented as mean ± s.e.mean, $n=7-33$ wells per bar from 3–5 separate experiments with RASMC obtained from four harvests. $^*P<0.05$ compared to wild-type; and $\#P<0.05$ compared to D-κB-Mut or D-κB-Del by ANOVA.

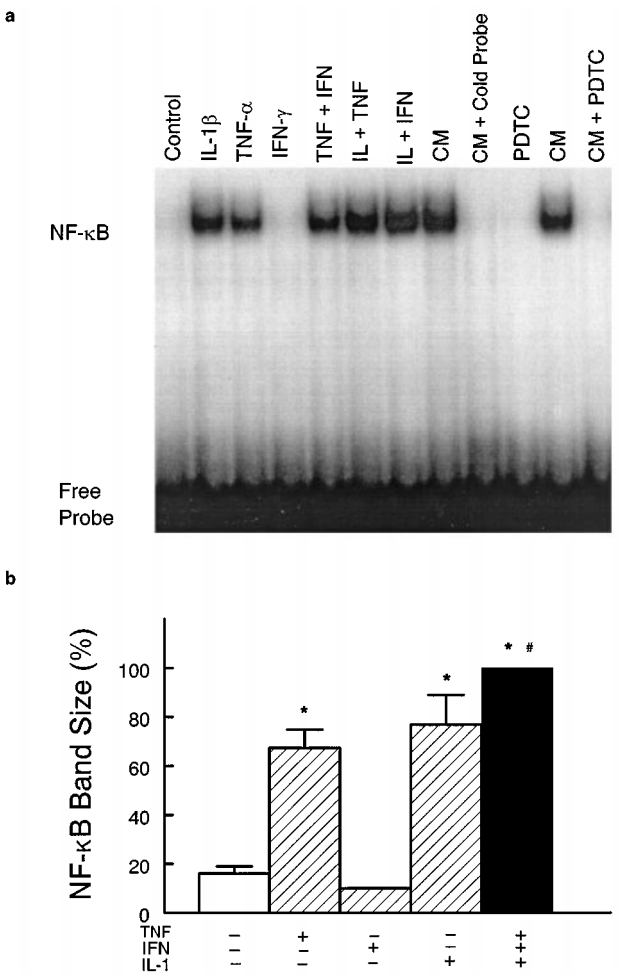

Figure 3 Cytokine induction of nuclear NF-κB DNA binding activity in RASMC by EMSA. RASMC were stimulated with individual cytokine or combinations for 30 min, and nuclear extracts were prepared. EMSA was performed by incubating an equal amount of the nuclear protein with ^{32}P-labelled NF-κB oligonucleotides. Bound and free oligonucleotides were separated by electrophoresis on a native gel. (a) Autoradiograph of nuclear NF-κB activity from RASMC in response to the stimulation of various cytokines. The figure represents two separate experiments with cells obtained from two harvests. (b) Quantification of nuclear NF-κB activity by phosphorimage. Data are expressed as percentage of maximal NF-κB activity by CM and presented as mean ± s.e.mean, $n=2-4$ wells per bar from four separate experiments with cells from three harvests. No significant difference between IL-1β and TNF-α; $^*P<0.05$, compared to control; and $\#P<0.05$, compared to TNF-α or IL-1β alone by ANOVA.

in which the 10 bp of the downstream or upstream NF-κB site or both were deleted (D-κB-Del, U-κB-Del, or DU-κB-Del, respectively. The mutants were constructed by changing the GGG residue sequence to CTC. These changes have been shown to generate a DNA sequence which no longer binds NF-κB (Perrella *et al*., 1996b). The mutant constructs, as well as the wild-type promoter, were transfected into RASMC and exposed to CM. The full wild-type promoter exhibited an average 29 fold induction (expressed as 100%, Figure 2) in response to CM, whereas in the downstream NF-κB mutants with either mutation (D-κB-Mut) or deletion (D-κB-Del) induction by CM decreased to 42–49% of the full wild-type promoter. Strikingly, when the upstream NF-κB site was mutated or deleted and transfected into RASMC, induction was decreased to 9–12% of the wild-type. The mutant with both NF-κB sites deleted showed only 7% of the full wild-type promoter activity (Figure 2). These data suggest that, unlike the mouse macrophage, the upstream NF-κB site is most important in mediating iNOS induction by cytokines in RASMC.

Activation of nuclear NF-κB binding by TNF-α without concomitant iNOS induction

TNF-α is a strong stimulant of NF-κB activity in many cell types (Schutze *et al*., 1995; Thurberg & Collins, 1998), and activation of NF-κB plays an important role in the induction of iNOS. However, TNF-α alone (even at 350 u ml^{-1}) did not stimulate the induction of rat iNOS promoter, protein or nitrite production in RASMC (Figure 1), suggesting that NF-κB activation is a necessary but not sufficient stimulus in iNOS induction. To confirm this, we studied nuclear NF-κB binding activities in response to different cytokines in RASMC (Figure 3). Resting RASMC did not exhibit significant nuclear NF-κB activity. The TNF-α, IFN-γ and IL-1β combination (CM) maximally activated nuclear NF-κB binding (Figure 3). The enhanced binding activity was abolished by excess cold oligo in the binding reaction (Figure 3a). Similarly, when cells were pre-incubated and subsequently stimulated in the presence of NF-κB inhibitor PDTC (100 μM) with CM, NF-κB binding activity was eliminated (Figure 3a). PDTC, at concentrations ranging from 50 to 200 μM has been shown to effectively inhibit nitrite production in A7r5 and C_6 gial cells (Pahan *et al*., 1999; Spink *et al*., 1995). Either TNF-α or IL-1β alone stimulated nuclear NF-κB activity to a similar extent reaching 67 and 76% of CM, respectively (Figure 3b). IFN-γ alone did not activate nuclear NF-κB (Figure 3). These data suggest that enhanced nuclear NF-κB activity alone is not enough to induce iNOS in RASMC.

The maximal induction of rat iNOS promoter requires more than two NF-κB binding sites in RASMC

To delineate the cytokine-responsive elements in the 5′-flanking region of the rat iNOS promoter, constructs with different lengths of the 5′-flanking region from the rat iNOS transcription starting site were generated and transfected into RASMC. Luciferase activities were measured following 6 h

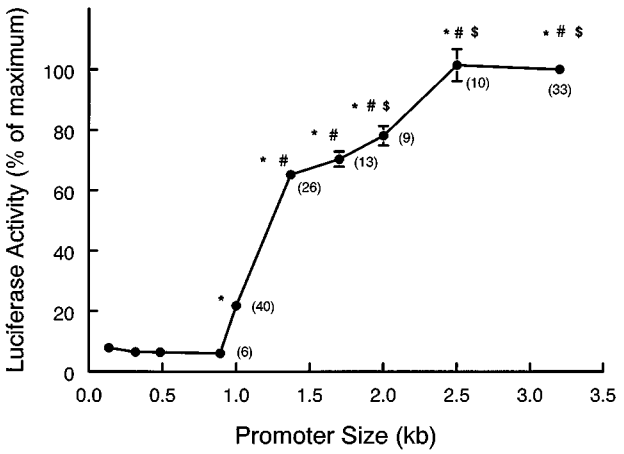

Figure 4 The maximal induction of rat iNOS promoter requires more than two NF-κB binding sites in RASMC. Deletion constructs with different lengths of the 5′-flanking regions of iNOS gene were generated and transfected into RASMC. Luciferase activity was analysed after stimulation with CM for 6 h. Data are expressed as per cent of full promoter activity (3.2 kb wild type) induced by CM and presented as means ± s.e.mean with number of wells per data point shown in parentheses, derived from 12 experiments with RASMC from seven separate harvests. $^*P<0.05$, compared to −893 bp; $\#P<0.05$, compared to −1000 bp; and $\$P<0.05$, compared to 1368 bp by ANOVA.

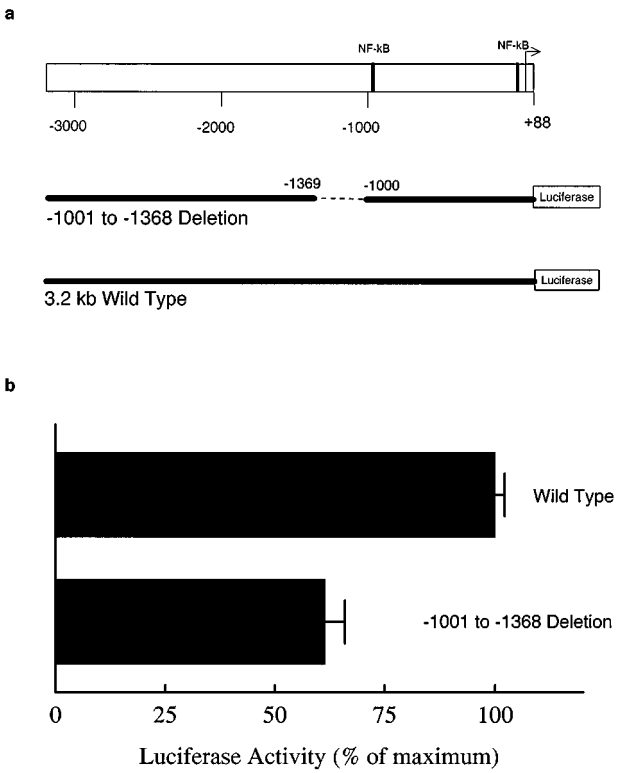

Figure 5 The effect of internal deletion of the fragment −1001 to −1368 bp on iNOS promoter induction in RASMC. (a) Schematic representation of internal deletion (−1001 to −1368 bp) constructs of the rat 3.2 kb iNOS promoter. (b) Wild-type or deletion constructs were transfected into RASMC, and luciferase activity was analysed after stimulation with CM for 6 h. Data are expressed as per cent of the promoter activity (3.2 kb wild-type) induced by CM and presented as means ± s.e.mean, $n=9$ wells per bar, obtained from three separate experiments with cells from three harvests. $^*P<0.05$, compared to wild type by ANOVA.

stimulation with CM. The construct containing a DNA fragment up to −893 bp from the transcription starting site showed very little response to CM (Figure 4). Three other constructs containing −135, −316 and −484 bp showed only basal activity in response to CM. A construct of up to −1000 bp, in which two NF-κB binding sites and all other known transcriptional factor binding sites, such as Oct-1, IRF-1 and GAS are included, showed significant activity to CM, but still was only 21.7% (s.e.mean ± 0.7) of the −3.2 kb wild-type (Figure 4). Promoter activity of 65.2% (s.e.mean ± 1.8) of the wild-type was observed in the construct of up to −1368 bp, and full promoter activity was observed in the −2493 bp construct (Figure 4). These data suggest that the two NF-κB binding sites, as well as other known binding sites located in the −1000 bp of the rat iNOS gene are necessary but not sufficient in conferring full promoter activity in RASMC. Regions of −1001 to −1368 and −2051 to −2493 bp contribute an additional 43 and 22% promoter activity, respectively (Figure 4).

Effect of internal deletion of the fragment between −1001 to −1368 bp on iNOS promoter activity in RASMC

To confirm the observation that the fragment between −1001 to −1368 bp contributes significant activity in response to CM stimulation, a construct with an internal deletion of the fragment from the 3.2 wild type promoter was created (Figure 5a) and transfected into RASMC. As expected, 40% of the full promoter activity in response to CM was lost in the internal deletion construct (Figure 5b).

Effects of orientation of the −1001 to −1368 bp fragment on iNOS promoter in RASMC

The orientation of the −1001 to −1368 bp fragment on promoter activity was studied since classic enhancer elements function independent of orientation (Atchison, 1988; Dynan, 1989). To generate the constructs with original or reverse orientation, the fragment between −1001 and −1368 bp was obtained by PCR, and ligated to the −1000 bp construct with blunt ends (Figure 6a). The correct orientation of the constructs was confirmed by sequencing. When the fragment was ligated to the −1000 bp construct in its original orientation, promoter activity was increased from 24% of full promoter activity in −1000 bp to 52% of the full promoter; this is very similar to the activity in the original −1368 bp construct. However, when the fragment was ligated to the −1000 bp construct in reverse orientation, the enhancement effect was abolished, and its activity was similar to the −1000 bp construct (Figure 6b). These data suggest that the enhancing activity of the −1001 to −1368 bp fragment is dependent on its orientation, and thus may not be a classic enhancer element.

Characterization of the −1001 to −1368 bp fragment in the rat iNOS promoter

Examination of the sequence of the −1001 to −1368 bp fragment revealed the presence of potential binding sites for three transcription factors that may be involved in the induction of iNOS. A GAS site, which is activated in response to IFN-γ stimulation is located at −1225 bp. At position −1126 bp is located a full copy of the AP-1 site, and at position −1026 bp, a SP-1 site, which may cooperate with NF-κB in activation of genes. In order to test the functional importance of these potential binding sites, mutants with

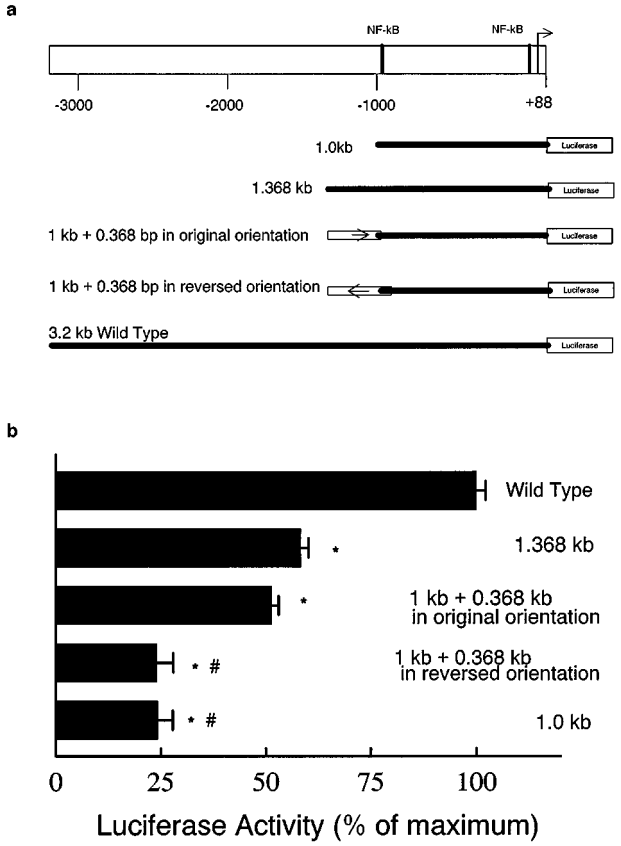

Figure 6 The effect of reversed orientation of the −1001 to −1368 bp fragment on induction of iNOS promoter activity in RASMC. (a) Schematic representation of the reversed orientation of −1001 to −1368 bp fragment of the rat iNOS promoter. (b) Activities of the wild-type and iNOS promoter with the reversed orientation of the 368 bp fragment in response to CM. Data are expressed as per cent of full promoter activity (3.2 kb wild-type) induced by CM and presented as means ± s.e.mean, $n = 3-12$ wells per bar from two separate experiments with cells obtained from two harvests. $^*P<0.05$, compared to wild-type; $\#P<0.05$, compared to −1368 bp or original orientation by ANOVA.

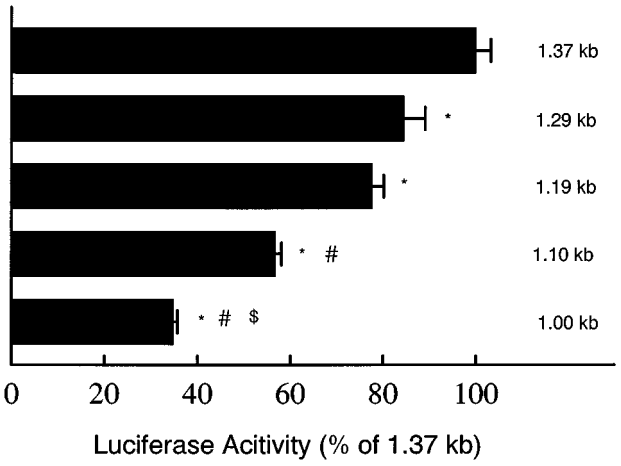

Figure 7 Deletion analysis of the 368 bp fragment on the induction of iNOs promoter activity. Deletion constructs with different lengths at −1001 to −1368 bp were generated and transfected into RASMC. Luciferase activity was analysed after stimulation with CM for 6 h. Data are expressed as per cent of the −1368 bp promoter activity induced by CM and presented as means ± s.e.mean, $n = 6-21$ wells per bar from three separate experiments with RASMC from two harvests. $^*P<0.05$, compared to the −1368 bp; $\#P<0.05$, compared to the −1191 or −1268 bp; and $\$P<0.05$, compared to the −1100 bp by ANOVA.

individual deletion of these three sites were created in a 1368 bp construct, and transfected into RASMC. None of the mutants affected promoter activity in response to CM. In order to delineate the elements responsible for iNOS induction in RASMC, serial deletion constructs of about 100 bp length each were generated at −1001 to −1368 bp. The activity of the constructs on iNOS promoter activity is shown in Figure 7. Promoter activity of the −1368 bp construct was expressed as 100% in response to CM, and progressively decreased to 85, 78 and 57% in constructs of −1286, −1191, and −1100 bp, respectively. The −1000 bp construct accounted for about 35% of the −1368 bp construct promoter activity, which is equivalent to 22% of full promoter activity.

Discussion

In the present study, we have shown that the upstream NF-κB binding site is functionally the most important regulatory site of iNOS induction in RASMC since it accounts for 90% of full promoter activity. The truncated construct of −893 bp, containing the downstream NF-κB binding site is not inducible by CM, but about 50% of full promoter activity is contributed by the downstream NF-κB binding site only when the upstream NF-κB binding site is present, suggesting that the downstream NF-κB binding site does not function independently. Taken together, these data indicate that the co-existence of two NF-κB binding sites is necessary for rat iNOS induction in RASMC. Thus, iNOS promoter regulation in RASMC is fundamentally different from that in the mouse macrophage iNOS, where the downstream NF-κB site is the most important element (Kim *et al*., 1997; Xie *et al*., 1994). The mouse construct of −1 to −85 bp is well induced, and the mutation in the downstream NF-κB binding site almost completely abolishes promoter activity, whereas mutation of the upstream NF-κB binding site still retains 50% of full promoter activity (Kim *et al*., 1997). The differences between murine and rat promoter regulation may be due to different cell types (macrophages vs vascular SMC) or different species (mouse vs rat). Perrella *et al*. have transfected the murine iNOS promoter into cultured rat primary SMC, and reported that a construct with −234 bp from the transcription starting site expresses almost full promoter activity in response to IL-1β (Perrella *et al*., 1996b). Mutation of the downstream NF-κB site in the full promoter (−1405 bp) decreased activity to about 45%. However, truncation of the mutant to −330 bp did not further decrease activity suggesting that the upstream NF-κB site contributed little to the activation of the murine iNOS promoter (Perrella *et al*., 1996b). These results differ from the present study in that, first, the 5′-flanking region of the rat iNOS gene up to −893 bp does not respond to CM, and second, the upstream NF-κB site is the most important element for promoter activity in the rat. Our result is similar to the observation by Spink *et al*. (1995) that upstream NF-κB site accounts for 66% of full promoter activity, but it is different in that there is no absolute requirement for the downstream NF-κB for effective promoter activity when mouse iNOS promoter is transfected into SMC line, A7r5. The difference may be due to either the mouse iNOS promoter and/or SMC line used by Spink *et al*. (1995).

Transcription factor NF-κB plays important roles in gene induction and in response to various stimuli. TNF-α is a well known stimulator of NF-κB. TNF-α, as well as IL-1β significantly stimulate nuclear NF-κB binding activity. However, TNF-α alone is not able to induce rat iNOS promoter activity, protein expression or nitrite production in

RASMC (Figure 1) suggesting that activation of additional transcription factors is required for iNOS induction in RASMC. Activation of STAT1α and IRF-1 by IFN-γ to bind GAS and IRF-E, and HMG-1(Y) by IL-1 to bind Oct have been reported to functionally or physically cooperate with NF-κB in inducing iNOS (Gao *et al*., 1997; Martin *et al*., 1994; Perrella *et al*., 1999; Spink & Evans, 1997). All these transcription factors are exclusively located within −1000 bp of the 5′-flanking region of the rat iNOS gene, which exhibits, however, only 22% of full promoter activity in response to CM. These data strongly suggest that additional unknown elements may be required to fully induce the iNOS gene in RASMC.

We have identified a region between −1001 to −1368 bp upstream of the transcription starting site that contributes about 40% of the full promoter activity. The enhancing activity of the region is dependent on the NF-κB sites, especially the upstream NF-κB site, since the enhancement is lost when the upstream NF-κB site is mutated. The function of the region seems to be orientation-dependent further suggesting that it may not be a classic enhancer element. Three potential transcription binding sites, GAS, AP-1 and SP-1, were individually deleted in that region, but the deletions did not significantly affect the activity of the promoter. Other undefined transcription factor(s) may bind to this region and contribute to the induction of the gene. It is possible that there are more than one undefined transcriptional binding site since deletion experiments have shown progressive decrease in promoter activity as the size of the 5′-deletions increased. These sites may function together and/or cooperate with NF-κB sites. Identifying the nature of these binding sites in this region is currently underway.

NO generated by iNOS in vascular smooth muscle cells has profound effects on the reactivity of the vessel wall and is believed to be a major cause of hypotension and vascular hyporeactivity in septic shock (Kilbourn *et al*., 1990; Liu *et al*., 1997; Titheradge, 1999; Wright *et al*., 1992). Accumulating evidence suggests that the transcriptional regulation of iNOS induction differs significantly among cell types and species. Understanding the complexity of iNOS induction in vascular SMC may provide the basis for development of agents for the management of septic shock.

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