Cloning and sequencing of the proximal promoter of the rat iNOS gene: activation of NFκB is not sufficient for transcription of the iNOS gene in rat mesangial cells

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Abstract It has previously been shown that expression of the inducible form of NO synthase (EC 1.14.23) is controlled at the transcriptional level and that induction of iNOS transcription is dependent on activation of transcription factors of the NFκB family. TNF-α and IL-1β synergistically stimulate iNOS transcription in rat glomerular mesangial cells. We have recently reported that endothelin-1 completely blocks cytokine-induced iNOS expression at the transcriptional level. To further investigate the molecular mechanisms and the role of NFκB in cytokine-elicited iNOS transcription, we cloned a 661 bp genomic rat DNA fragment, which contains 497 bp of the proximal iNOS promoter. An NFκB-binding site identical to that described for the murine sequence was identified and used for electrophoretic mobility shift experiments. We found that binding of NFκB is strongly induced in mesangial cells by both IL-1β and TNF-α. While endothelin-1 blocks cytokine-induced iNOS expression, it has no influence on the binding pattern of NFκB. We conclude from these data that transcription of iNOS in mesangial cells requires additional signals besides activation of NFκB.

Key words: iNOS regulation; iNOS promoter; Endothelin-1; NFκB activation; Oct-1; Mesangial cell; Rat kidney

1. Introduction

Considerable amounts of nitric oxide (NO) are produced by the inducible form of NO synthase in experimental glomerular disease [1]. NO is thought to function as an important mediator of the inflammatory response, e.g. by exerting toxic effects on glomerular cells or by affecting the apoptotic or proliferative cell phenotype. In fact, blocking of NO production by the NOS inhibitor, L-NMMA, has been shown to attenuate the severity of glomerular injury induced by anti-thymocyte serum [2]. Infiltrating activated macrophages release interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α), which induce the macrophage-type NO synthase (iNOS or NOS II) in mesangial cells (MCs) [3,4]. The induction of iNOS is regulated not only by these inflammatory cytokines and endotoxin but also by other factors, such as cAMP-elevating agents [5,6] and basic fibroblast growth factor [7]. Inhibitory effects have been shown for TGF-β, PDGF, IL-4, glucocorticoids, cyclosporin A, angiotensin II and endothelin-1 (ET-1). These studies were performed mainly in macrophages [8–10] as well as vascular smooth muscle cells (VSMCs) or MCs [11–17]. Most of the inhibitory effects occur at the transcriptional level. On the other hand, posttranscriptional and posttranslational effects, e.g. for TGF-β, have also been described. The published data show that the regulation of iNOS expression is highly cell and species specific. This renders the elucidation of a universal signaling pathway for iNOS regulation more difficult. Cloning of murine [18], human [19,20] and rat iNOS promoters has enabled us to examine transcriptional effects with authentic promoter fragments.

We have recently reported that ET-1 inhibits iNOS expression induced by TNF-α and IL-1β via the ET_A receptor in rat MCs [17]. Our data indicated that the inhibition occurs at the transcriptional level. The present study was performed to further improve our understanding of the molecular mechanisms involved in the inducing or inhibitory effects on iNOS expression.

2. Materials and methods

2.1. Reagents

Human recombinant IL-1β and ET-1 were purchased from Boehringer (Mannheim, Germany). Human recombinant TNF-α was a gift from BASF/Knoll (Ludwigshafen, Germany). DNA sequencing was performed with kits from Pharmacia (Freiburg, Germany) or Boehringer Mannheim. The cloning vector pBluescript KS^+ and all materials used for electrophoretic mobility shift assay (EMSA) as well as control oligonucleotides for NFκB and oct-1 were obtained from Stratagene (Heidelberg, Germany). Radioactive products and nylon blotting membranes were obtained from Amersham Buchler (Braunschweig, Germany). Tissue culture plastic was from Falcon (Becton-Dickenson, Heidelberg, Germany), media, sera and Taq polymerase from Gibco-BRL (Eggenstein, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany).

2.2. Cell culture

For preparation and culture of glomerular MCs from male Sprague Dawley rats, standard techniques were used, as described previously [21]. MCs were cultured in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamate, 5 ng/ml insulin, 100 U/ml penicillin and 1 mg/ml streptomycin (all from Sigma). To obtain quiescent cells, MCs were maintained in medium containing 0.5% FCS for 4 days before cytokine treatment. MCs were used between passages 8 and 19. In most experiments, the cytokines IL-1β and TNF-α were used at concentrations of 25 U/ml and 25 ng/ml, respectively.

2.3. Northern blot analysis

Using a murine cDNA clone kindly provided by Dr. Xie [18], iNOS RNA was detected by Northern blot hybridization as described previously [17]. For equivalent loading of the RNA probes, correction was carried out after rehybridization of the filter with a GAPDH probe.

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Abbreviations: ET-1, endothelin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin 1β; iNOS, inducible nitric oxide synthase; MCs, mesangial cells; NFκB, nuclear factor kappa B; PDTC, pyrrolidone dithiocarbamate; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor-α

The nucleotide sequence data reported in this article have been deposited in the GenBank data library (accession no. Z69839).

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2.4. Cloning and sequencing of the rat iNOS promoter

Genomic DNA from whole rat liver was prepared as described previously [22]. A genomic DNA fragment of 661 bp length was amplified by the polymerase chain reaction (PCR) technique using the primers 5′ CGGATCCTAGGCTAACAAGACCCAAG 3′ and 5′ ATCCTTTCTAGAAAACCTCC 3′ referred to as N5 and N6, respectively. The PCR product was extracted from an agarose gel [23] and cloned into the respective sites of the pBluescript KS^+ cloning vector (Stratagene). Sequencing of the cloned DNA (designated pINOSGEN2) was performed using a recently reported variant of Sanger's dideoxynucleotide chain termination method [24,25].

2.5. Electrophoretic mobility shift assay (EMSA)

1×10^7 MCs, were treated with combinations of cytokines/ET-1 or vehicle (see Section 3). Nuclear proteins were extracted as described by Schreiber et al. [26]. Routinely, 6 μg of the extract were used for each experiment. With pINOSGEN2 as template DNA and the primers 5′ AATGCATACAGACTAGG 3′ (N9) and 5′ GGTATTTATACCCATCC 3′ (N10), PCR was performed, to amplify a 127 bp DNA fragment containing a NFκB site (subsequently referred to as PCR127). Due to the lack of 5′-phosphate groups, this PCR product could be directly labeled with ^{32}P after purification by gel electrophoresis. For some experiments, a double-stranded oligonucleotide, representing a NFκB element with the sequence 5′ GATCGAGGGGACTTTCCCTAGC 3′, was labeled and used for the binding assay. The subsequent steps were performed following the recommendations of the distributor (Stratagene). To verify binding of the predicted nuclear protein, competition assays were performed with 5–100-fold molar excess of the unlabeled NFκB probe, unlabeled PCR127 or a double-stranded oligonucleotide which represents an oct-1 binding site (5′ GATCGAATGCAAAT CACTAGCT 3′).

3. Results

The transcription factor NFκB is thought to be one of the most important nuclear proteins involved in mediating cytokine-induced transcription of the macrophage-type iNOS. This was first shown in murine macrophages in gel shift and transient transfection studies [27]. As in macrophages, in MCs and other kidney cells, the induction of iNOS is inhibited by the NFκB inhibitor PDTC [28–30]. We have recently shown that ET-1 exerts a powerful inhibitory effect on cytokine-induced iNOS transcription in MCs [17]. To further elucidate this observation, we cloned a 637 bp genomic iNOS DNA fragment, which represents 497 bp of the rat iNOS promoter as well as 139 bp of exon 1. This was achieved by PCR, using primers which are homologous to known iNOS sequences from mouse (genomic) and rat (cDNA). Oligonucleotide N5 was a homologue to the 5′-region of the iNOS cDNA found in rat VSMCs [31] as well as in rat hepatocytes [32] and was extended with a *Bam*HI site. N6 was designed to match exactly the mouse promoter on position −427 to −408 [18]. N6 contains an intrinsic *Xba*I site of the murine iNOS promoter. The cloned rat DNA fragment was about 100 bp longer than expected in comparison to the murine promoter. Sequence analysis showed high homology to the previously cloned murine iNOS promoter (> 90%) apart from a gap of 93 bp in the upstream region (Fig. 1). In particular, all putative or presently established binding sites for transcription factors derived from the murine iNOS promoter were also found in the rat promoter.

Firstly, we examined the effects of IL-1β and TNF-α on the activation of NFκB using a synthetic, double-stranded oligonucleotide which represents a common NFκB consensus sequence. Nuclear extracts of MCs, which had been treated with IL-1β and TNF-α for 5 h showed enhanced binding of NFκB to the DNA probe compared to nuclear extracts obtained

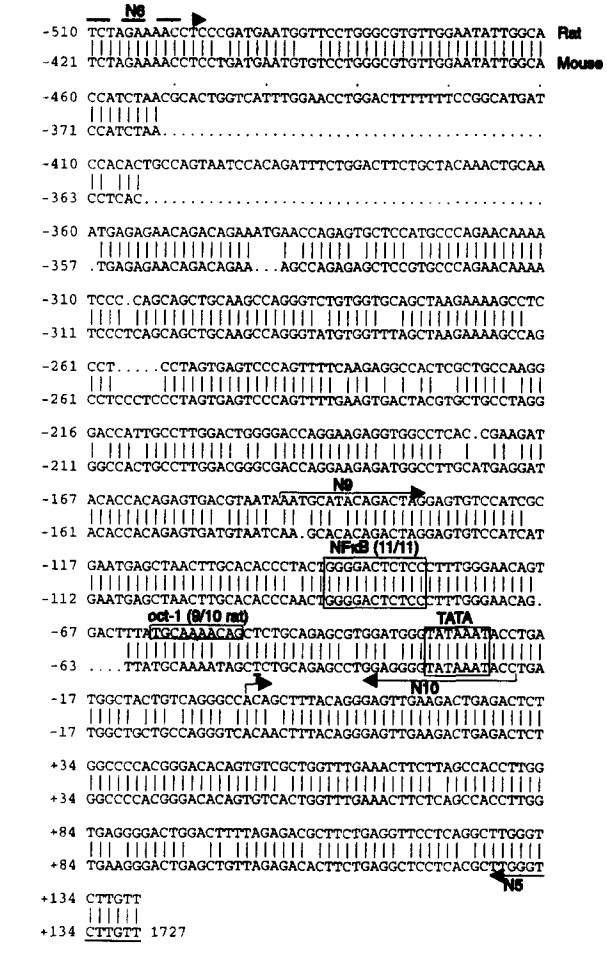

Fig. 1. Nucleotide sequence of the genomic clone, pINOSGEN2 in comparison to the respective murine sequence. The TATA box as well as the binding sites for oct-1 and NFκB, which were examined in this study, are boxed (for further details see [18]). The arrows indicate the primers N5 and N6 used to amplify the rat iNOS promoter from liver DNA. The sequence represented by N6 belongs to the murine iNOS promoter and does not necessarily exactly match the rat promoter (broken line). The arrows N9 and N10 represent the position of the primers, used to amplify a 127 bp fragment, which was used for EMSA.

from untreated MCs (Fig. 2). These results show that TNF-α and IL-1β induce translocation of active NFκB to the nucleus. Addition of a 5-fold molar excess of the unlabeled PCR product PCR127 (see Fig. 1) to the binding assay resulted in a clear competition, suggesting that the NFκB element in the proximal iNOS promoter, which has recently been described as essential for iNOS transcription [27], is able to bind its transcription factor.

To detect further nuclear proteins which bind to the proximal iNOS promoter region in MCs treated with cytokines or vehicle, we used the radiolabeled PCR fragment PCR127, which contains an oct-1 binding site besides the NFκB element mentioned above, as predicted by computer analysis (Fig. 1). As shown in Fig. 3, EMSA studies with nuclear proteins from MCs treated with TNF-α+IL-1β or vehicle revealed at least two different protein/DNA complexes. One of them, which is strongly induced by IL-1β and TNF-α, was characterized as the NFκB/DNA complex (Fig. 3b) as shown by competition experiments. The complex with lower retardation was identified as an oct-1/DNA complex by competition

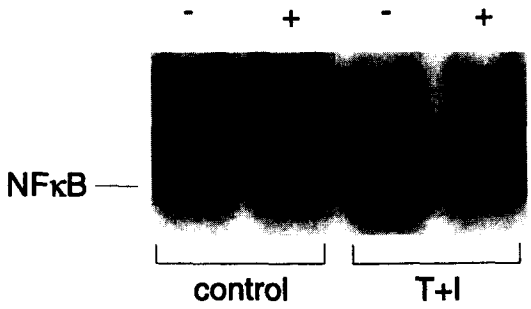

Fig. 2. Activation of NFκB in MCs treated with cytokines or vehicle. MCs were treated with TNF-α+IL-1β (T+I) or vehicle (control) for 5 h. Nuclear extracts were prepared and incubated with a radiolabeled oligonucleotide containing a NFκB consensus sequence. Partial competition was reached by adding a 5-fold molar excess of the unlabeled fragment PCR127 [+], which contains the proximal NFκB site of the rat iNOS promoter (see Section 2). The protein/DNA complexes were analyzed on a 8% PAA gel in 0.25×TBE buffer.

with an oct-1 representing double-stranded oligonucleotide (Fig. 3a).

As shown in Fig. 4, IL-1β and TNF-α have a strong synergistic effect on iNOS transcription in MCs, which is inhibited by ET-1. Inhibition of cytokine-mediated iNOS mRNA induction by ET-1 is due to transcriptional inhibition of iNOS expression since ET-1 does not reduce iNOS mRNA stability. The half-life of iNOS mRNA in the absence of ET-1 was 1.1 h (mean of two independent experiments giving similar data) and in the presence of ET-1, the half-life was 1.7 h (data not shown). This indicates that ET-1 does not accelerate iNOS mRNA degradation. IL-1β or TNF-α, when given alone, yield only weak or no detectable iNOS mRNA signals (Fig. 4a, upper panel). Binding of NFκB to the 127 bp radiolabeled DNA fragment PCR127 was induced after stimulation with TNF-α plus IL-1β, as found by EMSA. Addition of either TNF-α or IL-1β induced strong binding of NFκB to the iNOS promoter (Fig. 4a, lower panel). This finding suggests that translocation of NFκB to the nucleus is not sufficient for the transcription of iNOS. This is corroborated by the observation that ET-1 cannot change the binding pattern of NFκB elicited by TNF-α and by IL-1β (Fig. 4b), although ET-1 completely blocks cytokine-induced increases in iNOS mRNA steady-state levels.

4. Discussion

Among others, dexamethasone, aspirin and cyclosporin have been shown to inhibit NFκB binding to the promoters of some cytokine-inducible genes, including iNOS [33–40]. Different mechanisms have been considered to inhibit NFκB activation. The role of glucocorticoids has been studied most intensely. Their inhibitory effects on NFκB activation have been described to involve transcriptional activation of the physiological NFκB inhibitor, IκB [35,36], e.g. via direct inactivation of the p65 subunit of NFκB by the activated glucocorticoid receptor [37,38] or through changes in the redox state in the cell [39,40]. Since ET-1 does not lead to any changes in the binding pattern of NFκB, the data suggest that none of these effects plays a significant role in the inhibitory action of ET-1 on iNOS expression in MCs during time periods between 2 and 14 h. These periods were used for Northern blot analysis and EMSA, where ET-1 exerted a strong inhibitory effect on cytokine-induced iNOS mRNA expression but showed no detectable changes in the binding pattern of NFκB. Another consideration is that ET-1 could induce binding of a nuclear protein which occupies the NFκB site and consequently prevents binding of NFκB. Therefore, we decided to clone the proximal part of the iNOS promoter which contains a NFκB site. This enabled us to examine the binding of NFκB not only with a synthetic and established double-stranded consensus oligonucleotide, but also with an authentic fragment of the rat iNOS promoter where the NFκB site is embedded in the correct context.

As mentioned above, there are species-specific variations in the regulation of iNOS in MCs. Human iNOS is only ex-

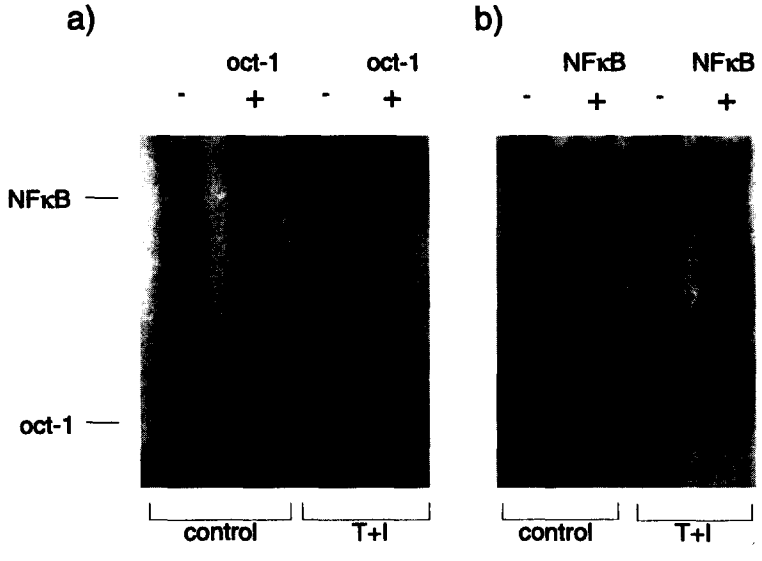

Fig. 3. Identification of an oct-1 and a NFκB element by competition analysis. MCs were treated with TNF-α (T) plus IL-1β (I) or vehicle for 5 h. Nuclear proteins were incubated with radiolabeled probe PCR127. Competition was performed with a 100-fold molar excess of an oct-1 (a) or a NFκB element (b) containing oligonucleotide [+].

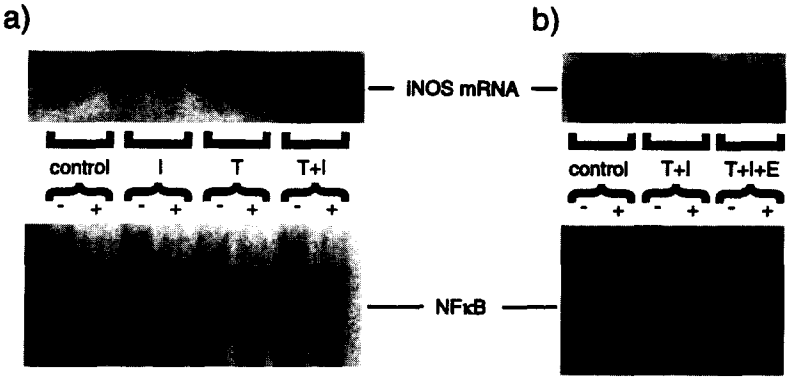

Fig. 4. Effects of TNF-α (T), IL-1β (I) and TNF-α+IL-1β (T+I) (a) or of TNF-α+IL-1β+ET-1 (T+I+E) (b) on iNOS transcription and binding of NFκB. MCs were treated with the indicated agents for 15 h (Northern blot) or 5 h (EMSA). Total mRNA was subjected to Northern blotting (upper panel) with an iNOS probe. Nuclear protein extracts were incubated with the radiolabeled fragment PCR127 and the complexes run on a 6% PAA gel (lower panel). Binding of NFκB was verified by competition with a 100-fold molar excess of the NFκB specific oligonucleotide.

pressed when combinations of endotoxin, interferon-γ, TNF-α and IL-1β are given as cocktails [41]. In contrast to rat MCs, iNOS is coinduced in murine MCs by the PKC activator PMA [42], which inhibits cytokine-induced iNOS expression in rat MCs (Fees, H. and Beck, K.-F., unpublished observations). Therefore, we considered the rat promoter as a useful tool to detect differences in the iNOS promoters, cloned so far [18–20].

Besides a NFκB site, the 127 bp proximal promoter fragment used in this study contains a putative TNF-RE and an interferon- responsive element (IRE), as described by Xie et al. [18]. Additionally, we could identify sequence homology to an oct-1 site. A nuclear protein binding to the respective promoter region is presumably constitutively expressed in rat MCs. Binding of active NFκB (presumably representing p50/50 and p50/p65 dimers) and the oct-1-protein could be demonstrated by competition experiments. The role of the TNF-RE remains unclear, but preliminary results using a DNA fragment representing this region suggest that binding to this element may be enhanced by ET-1 while it is not influenced by TNF-α. This divergence could explain the inhibitory effect of ET-1 on iNOS induction. This concept remains to be validated by further experiments. In summary, we conclude from our observations that cytokine-induced transcription of iNOS in rat MCs requires additional signals besides activation of NFκB.

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