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Transcriptional Regulation of the Human iNOS Gene by IL-1β in Endothelial Cells

Alexey Y. Kolyada and Nicolaos E. Madias

Department of Medicine, Tufts University School of Medicine, and the Division of Nephrology and the Tupper Research Institute, New England Medical Center, Boston, Massachusetts, USA

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Abstract

Background: Vascular endothelium participates in the control of vascular tone and function via the release of nitric oxide (NO) by the endothelial-type NO synthase (eNOS). Inducible NO synthase (iNOS) expression in endothelial cells occurs in many clinical conditions following induction by lipopolysaccharide or cytokines and generates large quantities of NO that result in endothelial cell activation and dysfunction. No information exists on the transcriptional regulation of the human iNOS gene (or that of other species) in endothelial cells.

Materials and Methods: We examined the transcriptional regulation of the human iNOS gene by interleukin-1β (IL-1β) in rat pulmonary microvascular endothelial cells (PVEC) by transient cotransfections of different iNOS-promoter constructs and cDNA of different transcription factors and regulatory proteins.

Results: The $-1034/+88$ bp iNOS promoter was strongly induced by IL-1β, the regulatory elements for such induction being localized downstream of -205 bp. Cotransfection experiments with NF-κB isoforms, IκB isoforms, and IKK mutants suggested that the NF-κB site at $-115/-106$ bp is important, but not sufficient, for induction of iNOS promoter and that the role of NF-κB is partially independent of its binding site. C/EBP sites within the $-205/+88$ bp region were shown to be responsible, along with NF-κB site, for induction of iNOS promoter by IL-1β. Overexpression of C/EBPα, C/EBPδ, and liver-enriched activator protein (LAP) activated the promoter, whereas overexpression of liver-enriched inhibitory protein (LIP) strongly suppressed it. C/EBPβ (LAP and LIP isoforms) was constitutively present in PVEC and was induced ($\sim$2-fold) by IL-1β, whereas C/EBPδ was not constitutively expressed but was strongly induced by IL-1β. Both C/EBPβ and C/EBPδ participated in DNA-protein complex formation.

Conclusion: Both NF-κB and C/EBP pathways are important for the transcriptional regulation of the human iNOS gene by IL-1β in PVEC.

Introduction

The vascular endothelium participates importantly in the paracrine control of vascular tone and function via the release of potent vasoactive molecules (1). Nitric oxide (NO) has been identified as the active component of endothelium-derived relaxing factor (EDRF) and represents a powerful vasodilator and antithrombotic substance (1,2). Just as other cells, vascular endothelial cells synthesize NO from the oxidation of the terminal guanidino nitrogen of L-arginine under the catalytic influence of the enzyme nitric oxide synthase (NOS) (2,3). Two NOS isoforms have been identified in endothelial cells, and these conform to two distinct functional classes: the endothelial-type NOS (eNOS) is constitutively expressed and requires calcium/calmodulin for activation; the inducible NOS (iNOS) is expressed following transcriptional induction and is calcium-independent (3). Each of the two isoforms is transcribed from a different gene. Basal synthesis of NO by eNOS in endothelial cells can be enhanced transiently by a number of agonists and physiologic stimuli resulting in the release of picomolar amounts of NO within seconds; eNOS-mediated NO production plays pivotal roles in the regulation of vascular tone and, hence, of blood pressure and flow, as well as the prevention of thrombus formation and the maintenance of vascular integrity (3). Deficiency of eNOS-mediated NO production is a critical feature of the syndrome of endothelial dysfunction.

On the other hand, iNOS expression in endothelial cells, just as in macrophages, vascular smooth muscle (VSM) cells, cardiomyocytes, and epithelial cells, requires several hours following transcriptional induction by bacterial lipopolysaccharide (LPS) or cytokines. Once synthesized, it catalyzes the sustained generation of large quantities of NO (3,4). High-output NO decreases DNA and protein synthesis and inhibits oxidative phosphorylation (3). Induction of endothelial iNOS and the resultant high-rate NO synthesis comprise an important component of the pathobiology of endothelial cell activation (5). This syndrome is triggered at variable intensity by many clinical conditions characterized by entry of microbial organisms and their products

Address correspondence and reprint requests to: Nicolaos E. Madias, M.D., Division of Nephrology, New England Medical Center, Box 172, 750 Washington St., Boston, MA 02111, USA. Phone: (617) 636-5895. Fax: (617) 636-1355. E-mail: nmadias@lifespan.org

into the blood stream; a surge of ambient cytokines, primarily TNF-α and IL-1β; or antibody-mediated induction of complement fixation at the endothelial cell surface. Among such conditions are sepsis, dialysis, cytokine therapy, ischemia-reperfusion injury, graft rejection, decompensated cirrhosis and the hepatorenal syndrome, vasculitis and other immune-mediated disorders, thrombotic microangiopathies, and regional inflammatory disorders (e.g., glomerulonephritis, hepatitis, arthritis, colitis) (5–8). Extensive evidence suggests that the sustained release of large quantities of NO from activated endothelial cells contributes importantly (along with similar release from surrounding cells, such as VSM cells, epithelial cells, and resident or invading inflammatory cells) to a host of pathophysiologic effects that are variably operative in the above conditions; these effects include profound vasodilation and hypotension, aggravation of inflammation, endothelial autocytotoxicity and lysis of neighboring cells, inhibition of eNOS expression, microvascular injury and consequent increased vascular permeability and organ failure, apoptosis of endothelial cells, killing or inhibition of microbial pathogens, and depression of cardiomyocyte contraction (3,5,7,9). Although NO itself inhibits adhesion and aggregation of leukocytes and platelets, leukocyte recruitment and a procoagulant state are hallmarks of the activated-endothelium phenotype as a result of the prevailing loss of microvascular integrity and other associated processes (5).

Atherosclerotic vessels are characterized by endothelial dysfunction, as defined by depressed endothelium-dependent vasodilation (10). Notably, human atherosclerotic lesions—but not normal vessels—feature widespread expression of iNOS in endothelial cells (as well as in VSM cells, macrophages, and mesenchymal-appearing intimal cells), which is likely induced by locally produced inflammatory cytokines (11). Indeed, atherosclerosis is now recognized as a chronic inflammatory process (12). A similar profile of iNOS expression has been identified in experimental transplant arteriosclerosis and other models of vascular injury (13). The pathophysiological significance of endothelial iNOS expression in these vascular lesions remains uncertain but such expression might impart both deleterious and beneficial effects.

There is currently no information on the transcriptional regulation of the human iNOS gene (or that of other species) in endothelial cells. The bulk of the work on the transcriptional regulation of the iNOS gene has involved the murine gene in macrophages and VSM cells (14,15). Corresponding work in the rat iNOS gene has been performed in mesangial cells (16) and cardiac myocytes (17). Regarding the human iNOS gene, we have recently described aspects of its transcriptional regulation in macrophages and VSM cells, and we have concluded that this regulation features considerable complexity and tissue specificity (18). This conclusion was amplified substantially by the work of others on several human epithelial cell lines (19–22). Thus, we reported that a construct of −1.1 kb of the 5′ flanking region of the human iNOS gene displayed LPS and cytokine inducibility in macrophages but not in VSM cells (18). Further, constructs larger than −4.7 kb of the human iNOS gene were required for cytokine inducibility in various human epithelial cells lines (20,22,23).

In the present study, we report the first information on the *cis*-regulatory elements responsible for the transcriptional activation of the human iNOS gene by IL-1β in endothelial cells. We show that these elements are localized within the −0.2-kb region of the human iNOS promoter, and we also demonstrate that transcription factors NF-κB, C/EBPβ, and C/EBPδ are involved in such activation.

Materials and Methods

Materials

Recombinant mouse IL-1β was obtained from R&D Systems (Minneapolis, MN). RPMI 1640 medium was purchased from Gibco-BRL, and fetal calf serum was obtained from HyClone. The Klenow fragment of DNA polymerase I and protease inhibitor cocktail were purchased from Boehringer Mannheim. Double-stranded poly(dI-dC) was from Pharmacia-LKB. Restriction enzymes were obtained from Gibco-BRL.

Cell Culture

Rat pulmonary microvascular endothelial cells (PVEC) used were a gift from Dr. Una Ryan (Boston University School of Medicine, Department of Medicine, Boston, MA). These cells have the morphologic characteristics of endothelial cells both by light and electron microscopy, and they express factor VIII antigen (24,25), and eNOS (AY Kolyada, NE Madias, unpublished observations). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin/streptomycin.

Plasmids

The plasmid phiNOS-1034Luc was generated by cloning the −1034 to +88 bp promoter fragment of the human iNOS gene between Xho I and Sma I restriction sites into the pGL2 plasmid (Promega) upstream of the reporter luciferase gene (18). The promoter for plasmid phiNOS-1034ΔNFκBLuc, which featured complete deletion of the NF-κB site at position −115 to −106 bp, was obtained by PCR using the phiNOS-1034Luc plasmid as template and appropriate primers (the oligonucleotide 5′-AGC TAA CTG TAC ACA AGA AGC TTT GGA AAC CAA AAA AAA A-3′, corresponding to −136 to −87 bp, was used as the coding primer). The mutant promoter was then cloned into the pGL2 vector. To generate the phiNOS-205ΔNFκBLuc plasmid, the mutant promoter was digested with Ban I and

the resulting fragment was recloned into the pGL2 vector. The phiNOS-183Luc, which had end-deletion of the C/EBP #4 site (at position −191 to −183 bp) was obtained by PCR using the phiNOS-1034 plasmid as template and the oligonucleotide 5′-ATG CGG TAC CAG CAA GAT CAG GTC ACC CAC A-3′ (corresponding to −183 to −162 bp) as the 5′-end primer. The phiNOS-183ΔNFκBLuc was constructed similarly using the phiNOS-1034ΔNFκB as template and the oligonucleotide 5′-ATG CGG TAC CAG CAA GAT CAG GTC ACC CAC A-3′ (corresponding to −183 to −162 bp) as the 5′-end primer. The plasmid phiNOS-183ΔNFκBΔC/EBP3,2Luc, which featured deletions of the NF-κB site (at position −115 to −106 bp) as well as the C/EBP#3 and C/EBP#2 sites (at positions −137 to −129, and −127 to −119 bp, respectively), was obtained by PCR using the phiNOS-183ΔNFκBLuc plasmid as template and appropriate primers (the oligonucleotide 5′-TGG CAG TCA CAG TCA TAA AAA GCT TTG GAA ACC AAA AAA A-3′, corresponding to −156 to −88 bp, was used as the coding primer). Finally, the plasmid phiNOS-77Luc was generated by PCR using the phiNOS-1034Luc plasmid as template and the oligonucleotide 5′-ACA CGG TAC CAA AAG AGA CCT TTA TGC AAA AAC AAC TC-3′ (corresponding to −76 to −51 bp) as the 5′-end primer.

Expression plasmids for the IκB kinase mutants, IKKα(K44A), and IKKβ(K44A) were kindly provided by Dr. D. Goeddel (26). Expression plasmids for IκBα, IκBβ, and IκBε isoforms were generously provided by Dr. D. Thanos (27), and expression vectors for the truncated isoforms of C/EBPβ, LAP*, LAP, and LIP were a generous gift of Dr. U. Schibler (28). The pCMVβ plasmid, expressing β-galactosidase under the control of the human cytomegalovirus early gene promoter, was purchased from Clontech.

Cell Transfection and Reporter Gene Assay

Cells were transfected by electroporation at 250V and 960 μF, using the Bio-Rad Gene Pulsar apparatus. We used a mixture of one of the human iNOS promoter constructs described above (with luciferase gene reporter) and the pCMVβ plasmid (in a ratio of 10:1) as an internal control of transfection efficiency. 5×10^6 cells per cuvette were transfected with 10 μg of luciferase plasmid and 1 μg of β-galactosidase plasmid in 0.5 mL DMEM with 10% FCS. After 16 hr, the media were changed. After an additional 8 hr had elapsed, IL-1β (10 ng/mL) was added. Cells were harvested 24 hr later, and extracts were prepared. The luciferase activity was determined using the Promega luciferase assay system and Monolight 2010 luminometer (Analytical Luminescence Laboratory). The β-galactosidase activity was determined as described (29). Transfections were performed in duplicate. In each protocol, at least three experiments were carried out. For co-transfection experiments, cells received a mixture of one of the human iNOS promoter constructs described previously (with luciferase gene reporter) and expression plasmids for different transcription factors or a corresponding empty vector. 5×10^6 cells per cuvette were transfected with 10 μg of luciferase plasmid and 10 μg of either expression plasmid or empty vector. In the case of transfection with different combinations of two expression plasmids, we used 10 μg of each of the two expression plasmids, or 10 μg of one of the expression plasmid and 10 μg of the empty vector. The results were normalized to protein concentration of cell extracts. Protein was measured by the Bio-Rad protein assay kit.

Western Blot Analysis

Cells were washed with PBS, scraped, lysed in lysis buffer with protease inhibitors, and centrifuged. The supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% and 12% gels were used for iNOS and C/EBP detection, respectively). Proteins were then transferred to Protran nitrocellulose membrane (Schleicher & Schuell). Membrane was blocked for 12 hr at 4°C with 3% skim milk in PBS. The blocked membrane was incubated for 1 hr in a primary antibody, washed 3 × 10 min in PBS with 0.05% Tween 20, incubated in the secondary peroxidase-conjugated antibody, and washed as above. Peroxidase activity was visualized using Western blot chemiluminescence reagent from NEN. Subsequently, the detection filter was washed in stripping buffer according to the manufacturer's procedure and restained with antibody against α-tubulin to control for variability of loading. Antibodies against iNOS (sc-650), C/EBPα (sc-61), C/EBPβ (sc-7962), C/EBPδ (sc-151), C/EBPε (sc-158), IκBα (sc-371), IκBβ (sc-945), and secondary antibodies conjugated with peroxidase were obtained from Santa Cruz Biotechnology. Monoclonal antibody against α-tubulin was obtained from Sigma.

Electrophoretic Mobility Shift and Competition Assay (EMSA)

The assay was performed as described previously (29). Briefly, 4–10 μg of cell protein extracts were preincubated for 15 min at 4°C in a reaction buffer that included double-stranded poly(dI-dC) in the presence or absence of 50-fold molar excess of competitor oligonucleotide. Radiolabeled double-stranded C/EBP4 oligonucleotide (30,000 cpm) was then added and the incubation continued for 30 min at 4°C. Free DNA and DNA-protein complexes were resolved on a 4% polyacrylamide gel in 1× TAE buffer. Following electrophoresis, the gel was dried and exposed to X-ray film. For supershift assays, antibodies against members of the C/EBP family and other transcription factors were included. In these cases, the reaction mixture was

preincubated for 40 min at room temperature. Antibodies against C/EBPα (sc-61), C/EBPβ (sc-150), C/EBPδ (sc-151), C/EBPε (sc-158), NF-κB p50 (sc-1190), NF-κB p65 (sc-109), and Oct-1 (sc-232) were obtained from Santa Cruz Biotechnology. Cell extracts were prepared as described previously for COS-1 cells (30). Briefly, cells from 90-mm dishes were harvested in PBS and pelleted by low-speed centrifugation. Cells were resuspended in 100 μl of a buffer containing 20 mM Tris/HCl (pH 7.4), 0.4 M KCl, 2 mM dithiothreitol, 10% glycerol, and a protease inhibitor cocktail. Cells were broken by freezing in liquid nitrogen and thawing on ice three times. Cell debris was removed by centrifugation at 4°C for 10 min in Microfuge, and the supernatant (whole cell extract) was aliquoted and stored at −70°C.

Nuclear extracts from untreated or IL-1β-treated (10 ng/mL for 15 min) PVEC cells were prepared essentially as described (31). Cells were lysed on ice in a buffer containing 10 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, a protease inhibitor cocktail, and 0.05% NP-40, and nuclei were pelleted by centrifugation. The crude nuclear pellets were extracted on ice in 50 μl of a buffer containing 20 mM HEPES (pH 7.4), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 0.5 mM dithiothreitol, and a protease inhibitor cocktail for 30 min. Membranes were removed by centrifugation and the nuclear extracts were stored at −70°C in small aliquots.

Results

The Region Downstream of −205 bp is Sufficient for Induction of the Human iNOS Promoter by IL-1β in Endothelial Cells

We have recently reported the molecular cloning of a 1.1-kb fragment containing the promoter region of the human iNOS gene and demonstrated its competence to promote luciferase gene transcription in VSM cells and macrophages (18). Contrary to VSM cells, however, IL-1β was a robust inducer of the human iNOS promoter activity in PVEC (mean ± SEM induction, 5.71 ± 0.77-fold in 24 independent experiments).

To localize the regulatory elements responsible for IL-1β induction, we examined the ability of sequential 5′-end deletion mutants of the human iNOS promoter to drive transcription of the luciferase gene in PVEC in the basal state and following treatment with IL-1β (Figure 1). A substantial reduction in the basal promoter activity occurred in constructs shorter than −122 bp (open bars) suggesting that the elements responsible for this activity reside within the −122/+88 bp region of the promoter. On the other hand, the level of induction of the promoter activity by IL-1β (hatched bars) was virtually identical among the −1034, −364, and −205 iNOS-luciferase constructs but dropped sharply in constructs shorter than −205 bp. These results indicate that the putative *cis*-regulatory elements responsible for IL-1β induction of the human iNOS promoter in PVEC are localized downstream of −205 bp.

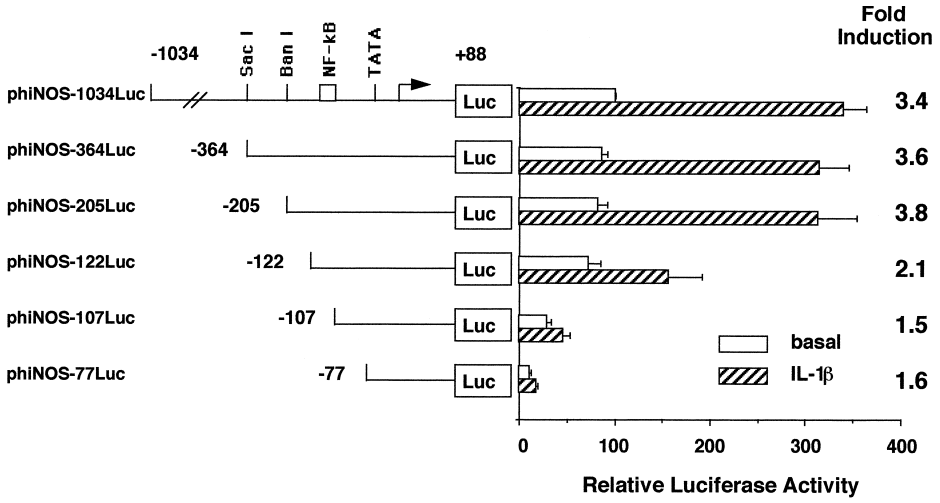

Fig. 1. Deletion analysis of the upstream 5′-flanking region (−1034 to +88 bp) of the human iNOS gene in PVEC. Designation of constructs is depicted on the left. A complete NF-κB element resides at position −115 to −106 bp. Open bars represent basal expression, and closed bars represent the effect of IL-1β, 10 ng/mL, added to culture medium for 24 hr. Luciferase activity is expressed relative to that achieved with the phiNOS-1034Luc plasmid under basal conditions. Luciferase activity was normalized to a cotransfected β-galactosidase internal standard. Values shown are the means ± SEM of five independent experiments. Fold induction, a measure of IL-1β inducibility of the human iNOS promoter construct, is expressed as the ratio of IL-1β-stimulated activity to basal activity. In a representative experiment, absolute values of luciferase activity in cells transfected with an empty pGL2 vector were $2.5 \times 10^4 \pm 0.06 \times 10^4$ RLU/$OD_{420} \times$ 1hr, and $2.6 \times 10^4 \pm 0.03 \times 10^4$ RLU/$OD_{420} \times$ 1hr in untreated cells and cells treated by IL-1β, respectively. In cells transfected with phiNOS-1034LUC plasmid, corresponding values were $136 \times 10^4 \pm 7.3 \times 10^4$ RLU/$OD_{420} \times$ 1hr, and $419 \times 10^4 \pm 23.6 \times 10^4$ RLU/$OD_{420} \times$ 1hr, respectively. Luciferase activity (expressed in relative luciferace units) was normalized by β-galactosidase activity (expressed as OD_{420} reached after incubation at 37°C for 1hr).

The NF-κB Element at −115 to −106 bp is Important for IL-1β Induction of the Human iNOS Promoter in PVEC

To evaluate the functional role of this NF-κB element in IL-1β induction of the human iNOS promoter in PVEC, we tested the transcriptional activity of the mutated construct phiNOS-1034ΔNF-κBLuc, which features complete deletion of the proximal NF-κB site (Figure 2). Deletion of the NF-κB element decreased the basal level of iNOS promoter activity by 20% and the IL-1β inducibility of the promoter by approximately 35%.

To demonstrate that the transcription factor NF-κB is translocating into the PVEC nucleus after IL-1β treatment, we performed EMSA using PVEC nuclear extracts and an oligonucleotide probe corresponding to the consensus sequence of NF-κB (Figure 12A). No DNA-NF-κB binding was detected in nuclear extracts from untreated cells (lane 2). Treatment of PVEC with IL-1β for 15 min induced a strong complex representing NF-κB (lanes 3 and 5). The specificity of this complex was demonstrated by competition with 100-fold excess of unlabeled oligonucleotide probe (lane 4). Further, supershift assays with antibodies against p50 (lane 6) and p65 (lane 7) indicated the presence of both p50/p50 and p50/p65 dimers of NF-κB in the formed DNA-protein complex Notwithstanding the decreases in basal activity and the IL-1β inducibility of the human iNOS promoter effected by deleting the proximal NF-κB element (Figure 2), attempts at transactivating the human iNOS promoter by overexpression of transcription factor NF-κB were unsuccessful (Figure 3). Cotransfection of PVEC with the phiNOS-205Luc construct and expression plasmids for both the p50 and p65 subunits of NF-κB did not affect the basal transcriptional activity of the human iNOS promoter; more strikingly, such an overexpression inhibited substantially the induction of the promoter by IL-1β (by approximately 40%) (Figure 3, first *vs* second set of bars). Additional cotransfection experiments, in which we employed either the p50 or the p65 expression plasmid, shed light into this result. Overexpression of the p65 subunit alone did not affect the basal level of the iNOS promoter activity but inhibited strongly (by approximately 65%) the IL-1β inducibility of the iNOS promoter (Figure 3, first *vs* third set of bars). On the other hand, overexpression of the p50 subunit alone augmented markedly both the basal activity of the human iNOS promoter and its activation by IL-1β, thereby limiting IL-1β inducibility to only 2.4-fold (Figure 3, first *vs* fourth set of bars). Further experiments suggested that the inhibitory effect of the p65 subunit on the IL-1β inducibility of the iNOS promoter could be attributed to IκBα activation. Transfection of PVEC with

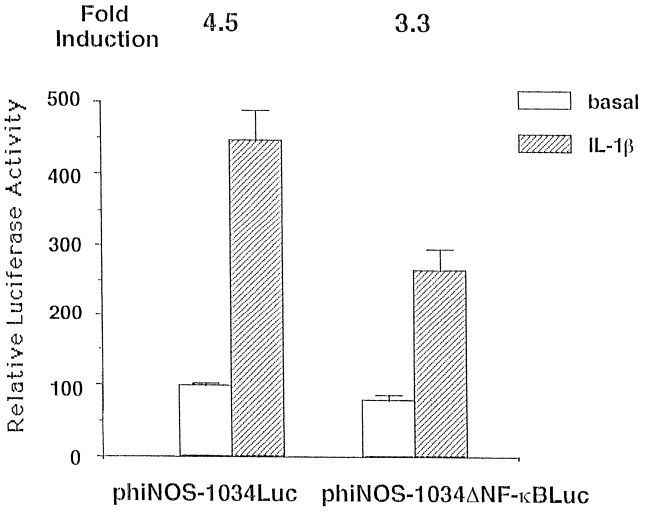

Fig. 2. Role of the NF-κB element at position −115 to −106 bp in the transcriptional activity of the human iNOS promoter in PVEC. The wild-type construct, phiNOS-1034Luc, is defined in Figure 1. The mutant construct, phiNOS-1034ΔNFκBLuc, features complete deletion of the NF-κB element at position −115 to −106 bp. Luciferase activity is expressed and normalized as in Figure 1. Values shown are the means ± SEM of five independent experiments.

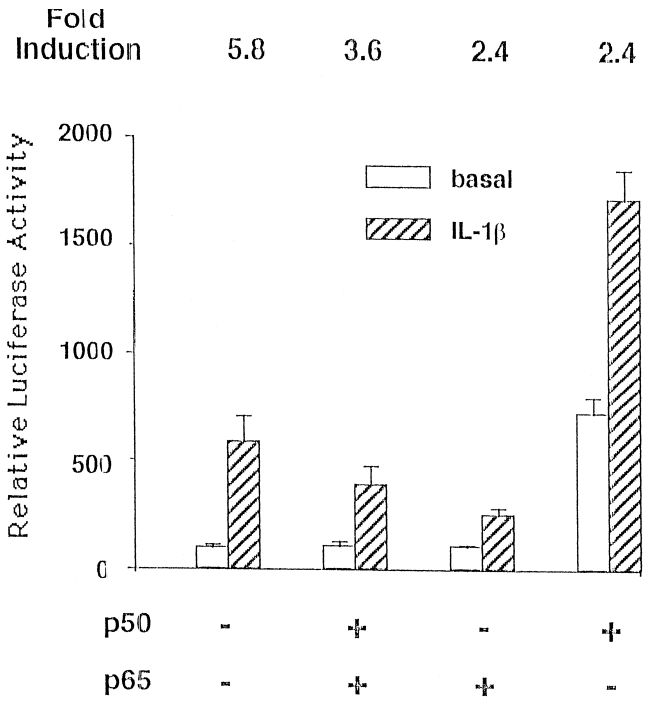

Fig. 3. Effect of overexpression of p50 and p65 subunits of transcription factor NF-κB on the transcriptional activity of the human iNOS promoter in PVEC. Cells were cotransfected with the phiNOS-205Luc plasmid, defined in Figure 1, and expression plasmids for either or both the p50 and p65 subunits of NF-κB, as depicted. Control cultures (first set of bars) received equal amounts of expression plasmids but without the cDNA of the p50 or p65 isoforms. Open bars represent the expression of luciferase reporter gene in untreated cells; closed bars represent effect of IL-1β treatment. Luciferase activity is expressed relative to that achieved in untreated PVEC cotransfected with the phiNOS-205Luc plasmid and an empty expression vector. Luciferase activity was normalized to protein concentration of cell extracts. Values shown are the means ± SEM of three independent experiments.

increasing amounts of p65 expression plasmid (1–5 μg) resulted in up-regulation of the IκBα, but not IκBβ protein at the 24-hr mark, as assessed by Western blot analysis (Figure 6A). Transfection of PVEC with increasing amounts of p50 expression plasmid (1–5 μg) did not result in up-regulation of IκBα or IκBβ proteins at the 24-hr mark (Figure 6B).

The Transcription Factor NF-κB Is Essential for Induction of the Human iNOS Promoter by IL-1β in PVEC But its Role Is Largely Indirect

Because our results suggested that overexpression of the p65 isoform inhibits IL-1β-mediated induction of the human iNOS promoter by limiting NF-κB availability, we carried out experiments designed to decrease the available level of this transcription factor by other methods. In a first approach, we cotransfected PVEC with the phiNOS-205Luc construct and expression plasmids of IκB kinase (IKK) mutants. As shown in Figure 4, cotransfection with these IKK mutants abrogated strongly the induction of the human iNOS promoter by IL-1β; this abrogation was particularly powerful in the case of the IKKβ(K44A) mutant, in which induction was denied fully. In a second approach for establishing a blockade of the NF-κB pathway, we overexpressed different IκB isoforms in cotransfection experiments. As depicted in Figure 5, cotransfection of PVEC with the phiNOS-205Luc construct and expression vectors of IκBα, IκBβ, or IκBε isoforms down-regulated strongly the IL-1β inducibility of the human iNOS promoter. In this regard, isoforms IκBα and IκBβ were equally effective, whereas IκBε displayed lower effectiveness.

In accord with the experiment presented in Figure 4, the Western blot depicted in Figure 6C indicated that blockade of the NF-κB pathway abrogates induction of the human iNOS promoter by IL-1β with sufficient power to down-regulate strongly expression of the iNOS gene at the protein level. iNOS protein was absent in untreated PVEC (lanes 1, 3, and 5). Treatment with IL-1β induced a strong iNOS band in control PVEC transiently transfected with an empty vector (without the cDNA of

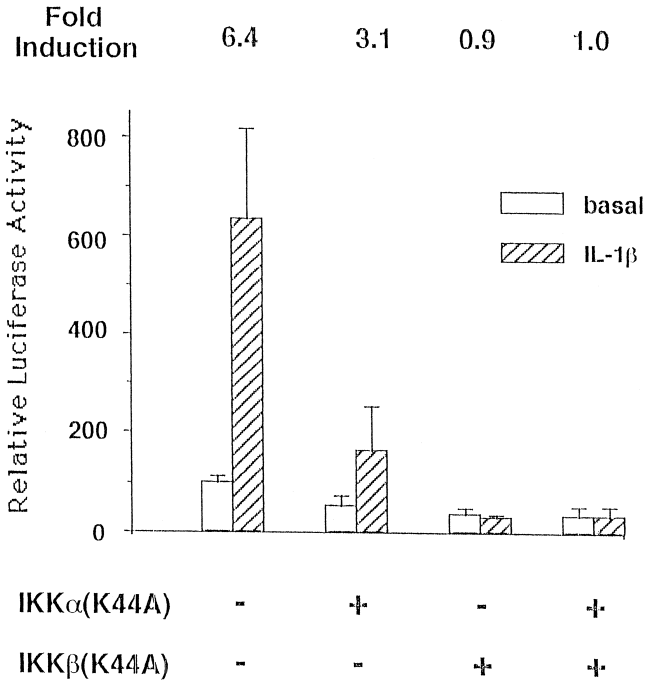

Fig. 4. Effect of IκB kinase mutants, IKKα(K44A), and IKKβ(K44A), on the transcriptional activity of the human iNOS promoter in PVEC. Cells were cotransfected with the phiNOS-205Luc plasmid, defined in Figure 1, and expression plasmids for either or both IκB kinase mutants, as depicted. Control cultures (first set of bars) received equal amounts of expression plasmids but without the cDNA of the IκB kinase mutants. Open bars represent the expression of luciferase reporter gene in untreated cells; closed bars represent effect of IL-1β treatment. Luciferase activity is expressed relative to that achieved in untreated PVEC cotransfected with the phiNOS-205Luc plasmid and an empty expression vector. Luciferase activity was normalized to protein concentration of cell extracts. Values shown are the means ± SEM of three independent experiments.

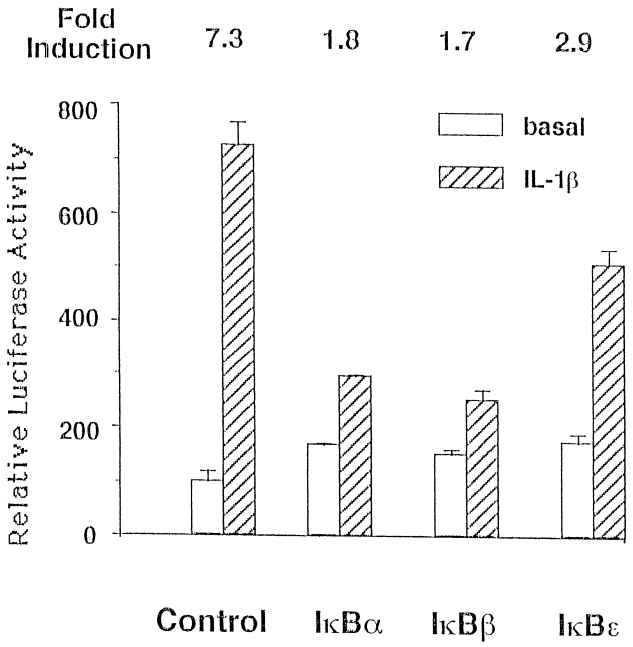

Fig. 5. Effect of overexpression of IκB isoforms on the transcriptional activity of the human iNOS promoter in PVEC. Cells were cotransfected with the phiNOS-205Luc plasmid, defined in Figure 1, and expression plasmids for the IκBα, IκBβ, or IκBε isoform. Control cultures (first set of bars) received equal amounts of expression plasmids but without the cDNA of anyone of the IκB isoforms. Open bars represent the expression of luciferase reporter gene in untreated cells; closed bars represent effect of IL-1β treatment. Luciferase activity is expressed relative to that achieved in untreated PVEC cotransfected with the phiNOS-205Luc plasmid and an empty expression vector. Luciferase activity was normalized to protein concentration of cell extracts. Values shown are the means ± SEM of four independent experiments.

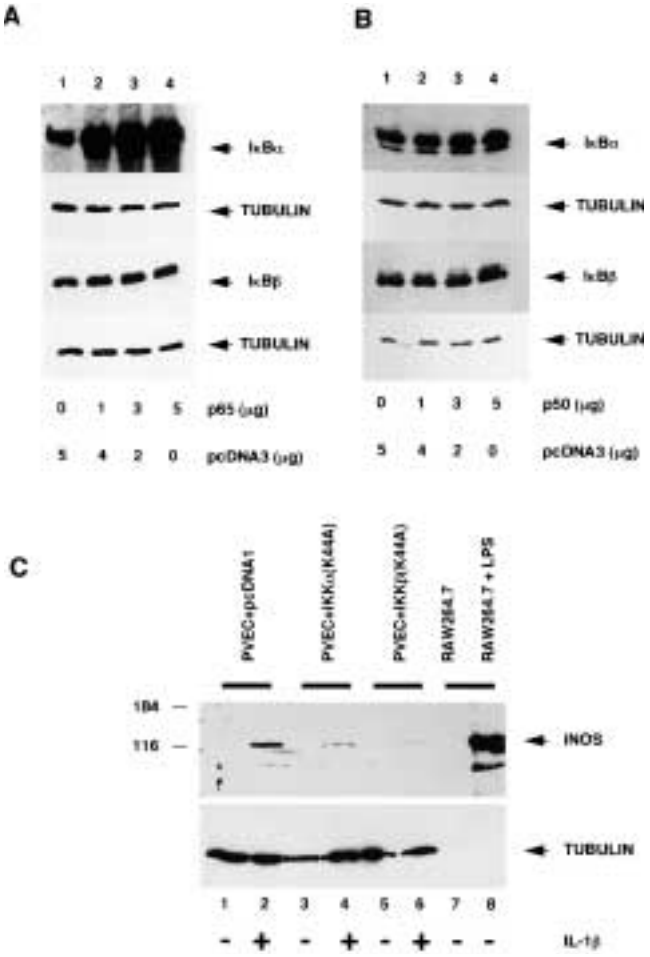

Fig. 6. Western blot analysis of transfected PVEC. (A–B) Effect of overexpression of increasing amounts of p65 plasmid **(A)** and p50 **(B)** on IκBα and IκBβ expression. PVEC were transfected with 1–5 μg of the expression plasmid or an empty vector as denoted at the bottom and harvested 24 hr after transfection. Cell lysate proteins were separated on a 7.5% acrylamide gel, transferred onto a membrane, stained with antibodies against IκBα or IκBβ, and then stripped and stained again with antibodies against α-tubulin to control for variability in loading. **(C)** Effect of IκB kinase mutants, IKKα(K44A) and IKKβ(K44A), on iNOS protein level in the presense or absense of IL-1β. Western blot analysis of PVEC transfected with empty vector (pcDNA1) or expression vectors for IKKα(K44A) or IKKβ(K44A) is shown. Twenty four hours after transfection, certain cell cultures were treated with IL-1β for another 24 hr, as denoted at the bottom of the figure. Filter was stained with an antibody against iNOS (sc-650), and then stripped and stained for a second time with an antibody against α-tubulin to control for variability in loading. Whole cell extracts prepared from RAW 264.7 cells that had been treated with LPS were used as a marker for iNOS protein. Molecular size markers (in kDa) are shown on the left.

IKK mutants, lane 2). By contrast, cells transfected with either IKKα (K44A) or IKKβ (K44A) expression vector expressed a much weaker (lane 4) or a nearly undetectable iNOS band (lane 6), respectively, following IL-1β treatment.

These results demonstrate clearly that transcription factor NF-κB is essential for activation of the human iNOS promoter by IL-1β in PVEC. Nonetheless, the strong abrogation of this induction by overexpression of IKKβ (K44A) or IκBβ (by 75% or greater, Figures 4 and 5) stands in stark contrast to the modest inhibition of IL-1β inducibility effected by complete deletion of the NF-κB site from the iNOS promoter (on the order of 35%, Figure 2). To explore further this discrepancy, we cotransfected PVEC with the IκBβ expression vector and either the wild-type phiNOS-205Luc construct or the mutant phiNOS-205ΔNF-κBLuc construct that features complete deletion of the resident NF-κB site. This deletion caused an approximate 40% inhibition of induction by IL-1β (Figure 7, first *vs* third sets of bars), which is similar in magnitude to the inhibition obtained by eliminating the NF-κB site in the 1.1-kb iNOS promoter construct (Figure 2). Overexpression of IκBβ suppressed the IL-1β inducibility of the wild-type promoter by approximately 65% (Figure 7, first *vs* second sets of bars) and that of the mutant promoter (devoid of NF-κB site) by approximately 45% (Figure 7, third *vs* fourth sets of bars). Similar

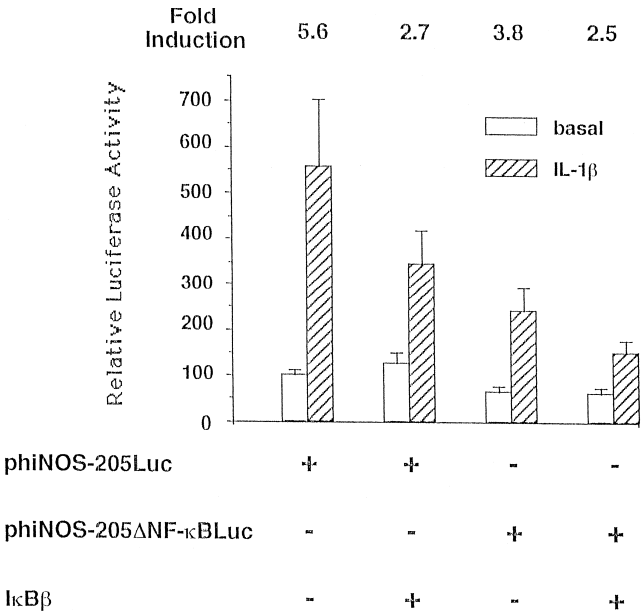

Fig. 7. Effect of overexpression of IκBβ isoform on the transcriptional activity of the wild-type human iNOS promoter and a mutant promoter featuring complete deletion of the NFκB site at position −116 to −105 bp in PVEC. The first two sets of bars represent PVEC cotransfected with the wild-type phiNOS-205Luc plasmid, defined in Figure 1. The remaining two sets of bars represent PVEC cotransfected with the mutant plasmid, phiNOSΔNFκBLuc. Cells of the second and fourth sets of bars also received an expression vector for IκBβ, whereas cells of the first and third sets of bars received an empty vector. Open bars represent the expression of luciferase reporter gene in untreated cells; closed bars represent effect of IL-1β treatment. Luciferase activity is expressed relative to that achieved in untreated PVEC cotransfected with the phiNOS-205Luc plasmid and an empty expression vector. Luciferase activity was normalized to protein concentration of cell extracts. Values shown are the means ± SEM of six independent experiments.

results were obtained in cotransfection experiments using the IKKβ (K44A) expression vector (data not shown). Taken together, these results reaffirm the functional importance of the NF-κB site in the activation of the human iNOS promoter by IL-1β in PVEC. They further indicate that the essential role of transcription factor NF-κB in this process is partially independent of its binding to the NF-κB site at position -115 to -106 bp. The data suggest either the presence of an unconventional, and heretofore unrecognized, NF-κB site in the human iNOS promoter or, more likely, the ability of the NF-κB factor to affect transcription of the iNOS promoter indirectly by interacting with other transcription factors.

C/EBP Sites Located Downstream of −205 bp are Important for IL-1β Mediated Induction of the Human iNOS Promoter in PVEC

On the basis of the findings presented in Figures 2 and 7, we concluded that approximately 30–40% of the induction of the human iNOS promoter by IL-1β is mediated via the NF-κB site, whereas the remainder depends on other *cis*-acting elements. To identify these regulatory elements, we subjected the $-205/+88$ bp region of the iNOS promoter to computer analysis that revealed 4 putative C/EBP binding sites arbitrarily designated as C/EBP site #1-4 starting from the proximal most site (Table 1). Compared with the consensus sequence of the C/EBP binding site, C/EBP sites #1 and #4 have each one mismatch, whereas sites #2 and #3 have each two mismatches. To evaluate the functional role of these putative C/EBP sites, we performed deletion analysis and compared the transcriptional activity of the derived mutants to the wild-type phiNOS-205Luc construct. As shown in Figure 8, deletion of the NF-κB site reduced IL-1β inducibility of the $-205/+88$ bp promoter construct by approximately 30% in accord with the findings presented above in Figure 7. A similar suppression of IL-1β inducibility was obtained when the most distal C/EBP site #4 was deleted ($-190/+88$ bp construct). Combined deletion of these two sites decreased the level of inducibility by 55%. Superimposing deletion of two additional C/EBP sites, #3 and #2, caused a 70% reduction in the level of promoter activation. Finally, generation of the $-77/+88$ bp construct, which featured deletion of all four C/EBP sites as well as the NF-κB site, suppressed the level of induction by more than 90%. Figure 8 also demonstrates that these successive mutations curtailed progressively the basal promoter activity to a level of only 20% of the wild-type construct. Together, these data indicate that the NF-κB site along with multiple C/EBP sites are largely responsible for both the basal activity of the human iNOS promoter and its activation by IL-1β.

To explore further the role of the C/EBP sites in the activation of the human iNOS promoter by IL-1β, we cotransfected PVEC with the phiNOS-205Luc construct and expression vectors of C/EBPβ isoforms. Liver-enriched transcriptional activator protein (LAP) is a truncated isoform of C/EBPβ that acts as a strong activator of transcription through the C/EBP

Table 1. C/EBP sites contained within the −205/+88 bp of the human iNOS promoter and oligonucleotides used in electromobility shift assays

C/EBP Sites	
Consensus	TTNNGNAAT
	G G
	−104 −96
Site #1	5′-TTTGGAAAC-3′
	−127 −119
Site #2	5′-GTACACAAG-3′
	−137 −129
Site #3	5′-TTAGCTAAC-3′
	−191 −183
Site #4	5′-TGATGTAAC-3′
C/EBP Oligonucleotides	
Consensus	5′-TGCAGATTGCGCAAT CTGCA-3′
Mutant	5′TGCAGAGACTAGTCT CTGCA-3′
C/EBP4	5′GCGACAGAG TGATGTAAC AGCAAG-3′

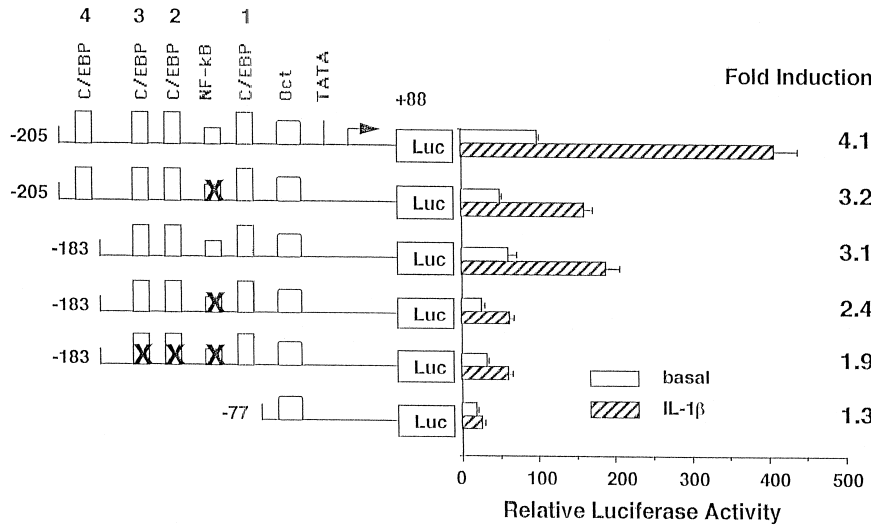

Fig. 8. Deletion analysis of the −205/+88 fragment of the 5′-flanking region of the human iNOS gene in PVEC. Designation of constructs is depicted on the left. Open bars represent basal expression and closed bars represent the effect of IL-1β, 10 ng/mL, added to culture medium for 24 hr. Luciferase activity is expressed relative to that achieved with the phiNOS-205Luc plasmid under basal conditions. Luciferase activity was normalized to a cotransfected β-galactosidase internal standard. Values shown are the means ± SEM of six independent experiments.

sites (28). Liver-enriched transcriptional inhibitory protein (LIP) is an even more truncated isoform of C/EBPβ that retains the DNA-binding domain but lacks the activation domain thereby acting as a strong dominant-negative repressor of C/EBP-mediated transcription (28). Overexpression of LAP up-regulated the basal activity of the −205/+88 bp promoter to a level similar of the IL-1β-mediated induction (Figure 9). Treatment with IL-1β could not elicit further activation of the promoter. By contrast, overexpression of LIP down-regulated markedly the basal level of promoter activity as well as its induction by IL-1β. These data support strongly the important role of C/EBP sites in IL-1β inducibility of the human iNOS promoter in PVEC.

The Transcription Factors C/EBPβ and C/EBPδ are Involved in the Activation of the Human iNOS Promoter by IL-1β in PVEC

The C/EBP (CCAAT/enhancer binding protein) transcription factors belong to the basic leucine zipper family of proteins and comprise a multigene family that currently includes six members (32). All members can dimerize in all intrafamilial combinations to generate a multitude of homo- or heterodimers that bind to the C/EBP cognate site on DNA (33). To gain insight into which ones of the C/EBP family members are effective transactivators of the human iNOS promoter, we cotransfected PVEC with the phiNOS-205Luc construct and expression vectors for C/EBPα, C/EBPβ, or C/EBPδ. As compared with the control state (i. e., cotransfection with an empty vector devoid of the cDNA insert), overexpression of C/EBPα or C/EBPδ increased the basal activity of the promoter by 8- to 9-fold, whereas overexpression of C/EBPβ caused a 2-fold increase (open bars, Figure 10). These results suggest that cotransfection with C/EBPα and C/EBPδ mimics induction by IL-1β. Treatment with IL-1β further up-regulated the promoter activity by 1.8- and 1.5-fold in the cases of C/EBPα and C/EBPδ, respectively, a substantially lower level of induction in comparison with the 4.9-fold up-regulation of the control state. These data further bolster the role of the C/EBP family members, and particularly C/EBPα and C/EBPδ, in the activation of the human iNOS promoter by IL-1β in PVEC.

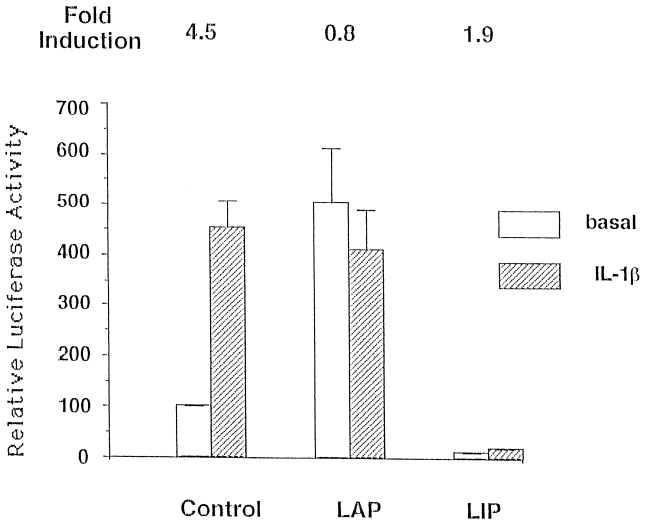

Fig. 9. Effect of overexpression of LAP (liver-enriched transcriptional activator protein) or LIP (liver-enriched transcriptional inhibitory protein) on the transcriptional activity of the human iNOS promoter in PVEC. Cells were cotransfected with the phiNOS-205Luc plasmid, defined in Figure 8, and an expression plasmid for LAP or LIP isoforms. Control cultures (first set of bars) received equal amounts of expression plasmids but without the cDNA of the LAP or LIP isoforms. Open bars represent the expression of luciferase reporter gene in untreated cells; closed bars represent effect of IL-1β treatment. Luciferase activity is expressed relative to that achieved in untreated PVEC cotransfected with the phiNOS-205Luc plasmid and an empty expression vector. Luciferase activity was normalized to protein concentration of cell extracts. Values shown are the means ± SEM of four independent experiments.

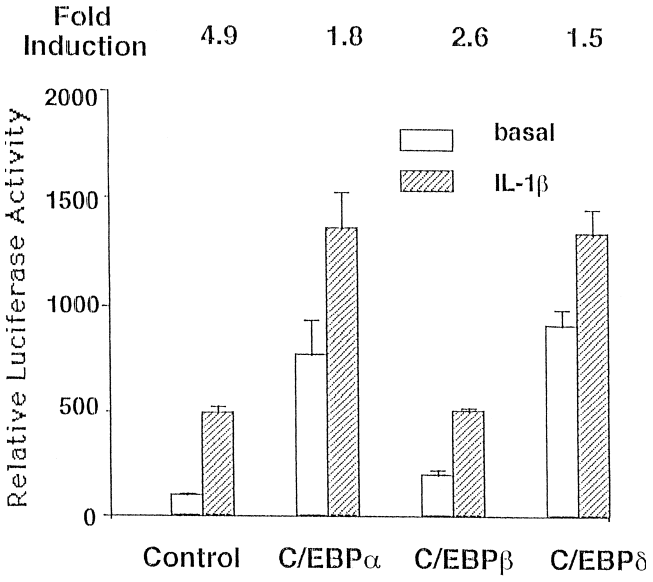

Fig. 10. Effect of overexpression of members of the C/EBP family of transcription factors on the transcriptional activity of the human iNOS promoter in PVEC. Cells were cotransfected with the phiNOS-205Luc plasmid, defined in Figure 8, and an expression plasmid for C/EBPα, C/EBPβ or C/EBPδ. Control cultures (first set of bars) received equal amounts of expression plasmids but without the cDNA of C/EBPα, C/EBPβ or C/EBPδ. Open bars represent the expression of luciferase reporter gene in untreated cells; closed bars represent effect of IL-1β treatment. Luciferase activity is expressed relative to that achieved in untreated PVEC cotransfected with the phiNOS-205Luc plasmid and an empty expression vector. Luciferase activity was normalized to protein concentration of cell extracts. Values shown are the means $\pm$ SEM of three independent experiments.

Next we addressed the question of which ones of the C/EBP family members are expressed in PVEC. Western blot analysis revealed that C/EBPα and C/EBPε are not expressed in these cells, at least at the sensitivity level of the method (data not shown). Regarding C/EBPβ, this transcription factor comprises at least four isoforms: LAP* (full-length C/EBPβ, 38 kDa), LAP (35 kDa), LIP (21 kDa), and a 14-kDa isoform (34). These C/EBPβ isoforms are produced by the process of alternative translation through a leaky ribosome scanning mechanism that leads to initiation at downstream AUG codons and yields N-terminally truncated products (28). As a result of the N-terminus truncation, both LIP and the 14-kDa isoform lack most of the transactivation domain (28). The activity of C/EBPβ as transactivator depends on the prevailing ratio between large-size isoforms (LAP* and LAP) and truncated isoforms (LIP and 14-kDa) (28).

When C/EBPβ was overexpressed in COS-1 cells or in PVEC, both activator isoforms (LAP* and LAP), and dominant negative isoforms (LIP and 14-kDa isoform) were identified by Western blot analysis (data not shown). In untransfected and untreated PVEC, however, endogenous C/EBPβ was represented by a weak band with a molecular weight intermediate between LAP* and LAP, a strong band corresponding to LAP, and a third band corresponding to LIP. The 14-kDa isoform was not identified in these cells. Treatment with IL-1β up-regulated the LAP and LIP bands by approximately 2-fold (Figure 11A, lane 3). Because the ratio between these activator and repressor isoforms did not change appreciably after IL-1β treatment, we conclude that C/EBPβ is likely not the main transactivator of the human

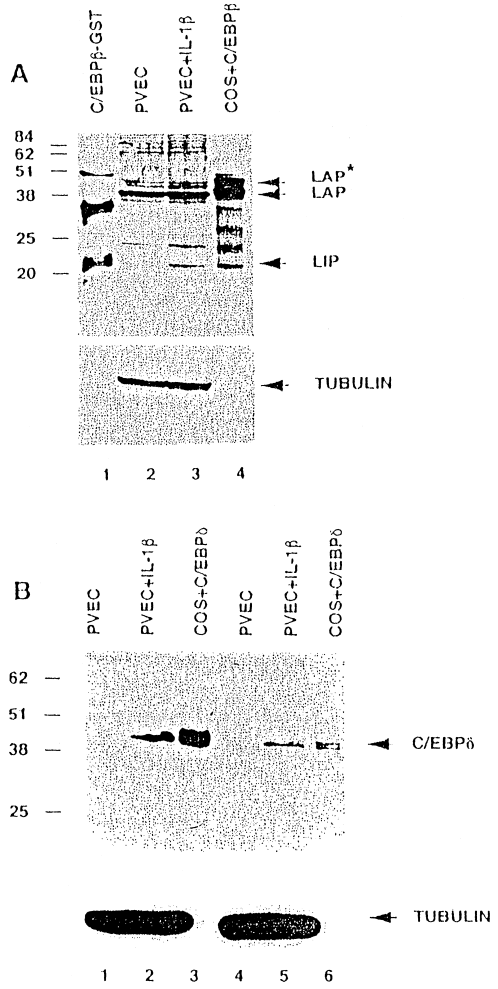

Fig. 11. Western blot analysis of C/EBP proteins in PVEC. **(A)** Lanes 2 and 3 represent whole cell extracts from untreated PVEC and PVEC treated with IL-1β for 24 hr, respectively. Lane 1 represents C/EBPβ-GST fusion protein expressed in bacteria and lane 4 represents whole cell extract from COS-1 cells transfected with C/EBPβ expression plasmid. Filter was stained with antibody against C/EBPβ (sc-7962), and then stripped and stained for a second time with an antibody against α-tubulin to control for variability in loading. Molecular size markers (in kDa) are shown on the left. **(B)** Whole cell extracts obtained in two independent experiments from untreated PVEC (lane 1 and 4), PVEC treated with IL-1β for 24 hr (lane 2 and 5), and COS-1 cells transfected with C/EBPδ expression plasmid (lane 3 and 6) were stained with antibody against C/EBPδ (sc-151). Filter was then stripped and stained for a second time with antibody against α-tubulin to control for variability in loading. Molecular size markers (in kDa) are shown on the left.

iNOS promoter in response to IL-1β treatment in PVEC. Unlike C/EBPβ, C/EBPδ was not detected in untreated cells (lanes 1 and 4) but was expressed in abundance after IL-1β treatment (lane 2 and 5) (Figure 11B). These results support a role of C/EBPδ in the activation of the human iNOS promoter by IL-1β in PVEC.

The nature of the C/EBP family members that bind to C/EBP sites of the human iNOS promoter was assessed by EMSA using crude PVEC extracts and an oligonucleotide probe corresponding to C/EBP site #4 of the promoter (Table 1 and Figure 8). As can be seen in Figure 12, extracts of untreated cells did not form any complexes with the labeled probe (lane 2), whereas a strong DNA-protein complex appeared after treatment of PVEC with IL-1β for 24 hr (lane 3). This complex was competed out completely by an excess of cold C/EBP site #4 oligonucleotide (lane 6) as well as the consensus C/EBP oligonucleotide (lane 4) but not a mutated C/EBP oligonucleotide (Table 1) (lane 5). Further, this complex was not supershifted by antibodies against C/EBPα or C/EBPε (lanes 7 and 10, respectively), whereas antibodies against C/EBPβ or C/EBPδ formed complexes that aggregated at the origin thereby attenuating the dominant complex (lanes 8 and 9, respectively). Quantitation analysis showed that the amount of the DNA-protein complex was reduced by 80% and 51% by antibodies against C/EBPβ and C/EBPδ, respectively (average of 5 experiments). No such supershifting effect was detected when antibodies against transcription factors Oct-1, p50, or p65 were used (lanes 11, 12, and 13, respectively).

Discussion

The results of the present study indicate that the *cis*-regulatory elements responsible for the transcriptional activation of the human iNOS gene by IL-1β in endothelial cells are localized in the 5′-flanking region downstream of −0.2 kb. Further, our data demonstrate that such induction depends on transcription factors NF-κB, C/EBPβ, and C/EBPδ, cognate sequences for these factors residing within the −0.2-kb region of the human iNOS promoter. These data provide new insights into the themes of complexity and tissue specificity that characterize the transcriptional regulation of the human iNOS gene.

Blockade of the NF-κB pathway abrogated strongly the IL-1β-mediated induction of the human

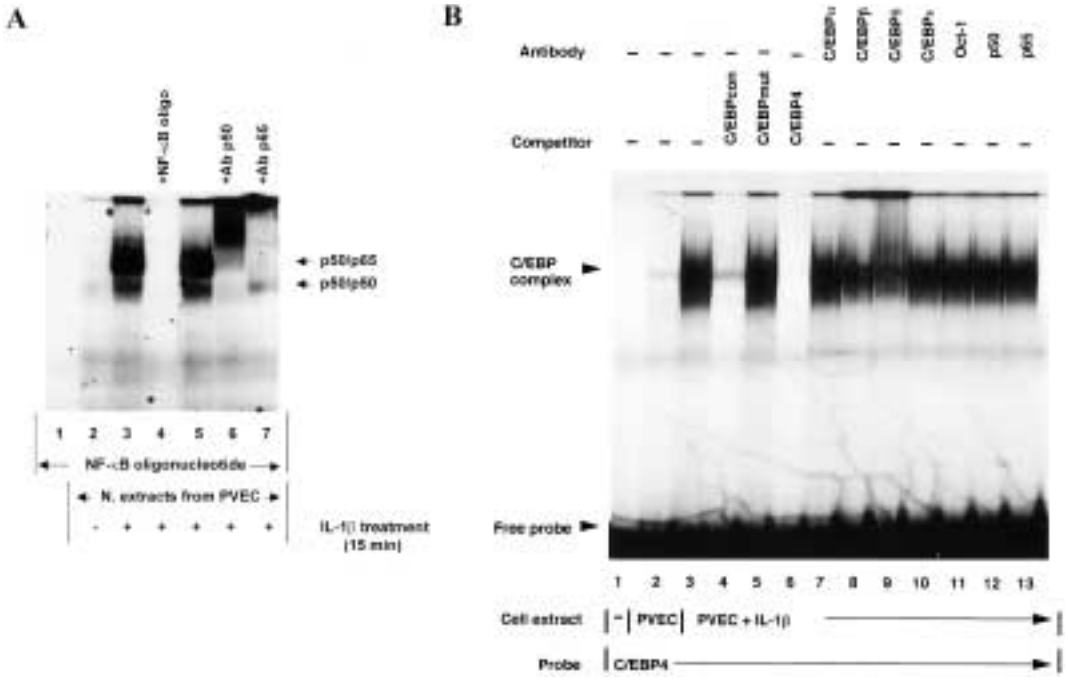

Fig. 12. Electrophoretic mobility shift and competition assays. (A) EMSA with NF-κB consensus oligonucleotide (5′-TTA GAG GGG ACT TTC CGA GAG-3′) as a probe and PVEC nuclear extracts. Lane 1 contains labeled oligonucleotide but no extracts. Labeled oligonucleotide was incubated with extracts from untreated PVEC (lane 2) or PVEC treated with IL-1β for 15 min (lanes 3 to 7). Lane 4 contains competitor "cold" oligonucleotide at 100-fold molar excess relative to the labeled oligonucleotide probe. Lanes 6 and 7 contain antibodies against p50 or p65 subunits of NF-κB transcription factor, as denoted at the top of the figure, and represent supershift assays. Positions of p50/p50 and p50/p65 complexes are denoted on the right. **(B)** Electrophoretic mobility shift and competition assays using cellular extracts from PVEC and C/EBP4 oligonucleotide corresponding to C/EBP site #4 (−191 to −183 bp) of the human iNOS promoter as binding probe. Labeled oligonucleotide was incubated with extracts from untreated PVEC (lane 2) or PVEC treated with IL-1β for 24 h (lanes 3 to 13). Lane 1 contains labeled oligonucleotide but no extracts. Lanes 4, 5, and 6 contain competitor "cold" oligonucleotides at 50-fold molar excess relative to the labeled oligonucleotide probe (consensus C/EBP oligonucleotide in lane 4, mutated C/EBP oligonucleotide in lane 5, and C/EBP4 oligonucleotide in lane 6, as described in Table 1). Lanes 7 to 13 contain antibodies against different transcription factors, as denoted at the top of the figure, and represent supershift assays.

iNOS promoter and protein in PVEC (Figures 4–6). Notwithstanding, simultaneous overexpression of the p50 and p65 subunits of transcription factor NF-κB failed to up-regulate, and indeed inhibited, the induction of the human iNOS promoter by IL-1β (Figure 3). In point of fact, overexpression of the p65 subunit alone down-regulated substantially the IL-1β inducibility of the human iNOS promoter, whereas overexpression of the p50 isoform up-regulated strongly both the basal activity and the IL-1β-induced activation of the promoter. Regarding this inhibitory effect of the p65 subunit, the IκBα promoter contains a NF-κB site and promoter activity can be enhanced by overexpression of transcription factor NF-κB, especially the p65 subunit (35,36). Indeed, we found that overexpression of the p65 subunit in PVEC led to a strong up-regulation of the IκBα protein. We interpret our results to indicate that the level of expression of the p50 subunit of NF-κB, but not that of the p65 subunit, is crucial for the induction of the human iNOS promoter by IL-1β in PVEC. We hypothesize that the activation of the promoter is mediated by p50/p65 dimers. If availability of the p50 subunit is a limiting factor in PVEC, its overexpression can enhance induction of the human iNOS promoter by IL-1β. On the other hand, up-regulation of the p65 subunit might result in formation of p65/p65 dimers. Such dimers can be particularly effective in IκBα up-regulation, thereby serving as a feedback mechanism that prevents overstimulation of the iNOS promoter by NF-κB. Our results demonstrate the functional importance of the proximal NF-κB element residing at −115 to −106 bp for induction of the human iNOS promoter by IL-1β in PVEC. This element corresponds completely to the consensus sequence of the NF-κB binding site and its deletion reduces IL-1β-mediated induction of the iNOS promoter by 30–40% (Figures 2, 7, and 8). But even a mutant promoter devoid of the NF-κB site responds to NF-κB depletion with strong suppression of IL-1β-mediated induction of the iNOS promoter (Figure 7). These results raise the possibility of an atypical NF-κB site in the human iNOS promoter that heretofore remains unidentified. However, an alternative and more plausible explanation of these findings is that the stimulation of IL-1β-mediated induction of the iNOS promoter by transcription factor NF-κB is largely indirect and reflects its interaction with other transcription factors.

It remains unclear how overexpression of IκB isoforms and IKK mutants in PVEC blocks IL-1β-mediated induction of the iNOS promoter when the NF-κB site has been rendered inactive by mutations. One possibility is that this effect is mediated indirectly through C/EBP sites, as it is the case with the alpha-1 acid glycoprotein (AGP) promoter (37). The p65 subunit of NF-κB has been shown to interact with the C/EBPβ and C/EBPδ proteins and such protein-protein interaction augments activation of the AGP promoter by C/EBP (38). Therefore, it is plausible that some members of the NF-κB and C/EBP families of factors interact with each other in PVEC and such an interaction is essential for iNOS promoter up-regulation; in turn, blockade of NF-κB influx in the nucleus by overexpression of IκB isoforms and IKK mutants will prevent activation of the iNOS promoter by C/EBP proteins. On the other hand, the participation of the p65 or p50 subunits in this interaction was questioned by our EMSA data showing no supershift effect of antibodies against the p65 or p50 proteins on the complex between nuclear proteins and the C/EBP oligonucleotide probe (Figure 12, lanes 12 and 13). Nonetheless, it is possible that the interaction between members of the NF-κB and C/EBP families in such a complex is not sufficiently stable to be detected under the conditions of our EMSA.

An additional possibility might be that C/EBP expression in PVEC is mediated by NF-κB. In that case, blocking the NF-κB pathway would cause down-regulation of C/EBP. We found, however, that transfection of IKKα(K44A) or IKKβ(K44A) mutants in PVEC did not down-regulate C/EBP expression following IL-1β treatment, as defined by EMSA (*data not shown*).

In a recent report, we showed that the 1.1-kb 5′-flanking region of the human iNOS gene was induced strongly by LPS and INF-γ and modestly by IL-1β in macrophages, but not in VSM cells (18). In addition, we demonstrated the marked functional importance for LPS-inducibility in macrophages and basal transcription in VSM cells of the proximal NF-κB element residing at −115 to −106 bp. Importantly, the proximal NF-κB element at position −85 bp in the murine iNOS promoter confers LPS inducibility in macrophages (14) and the same element at position −97 bp in the rat iNOS promoter is essential for IL-1β-induced activation of the promoter in mesangial cells (16). Subsequent work by other investigators indicated that constructs of the 5′-flanking region of the human iNOS gene longer than −4.7 kb were required for cytokine inducibility in human liver (AKN-1) and human lung (A549) epithelial cell lines (19,20) and longer than −8.7 kb in a human colorectal (DLD-1) epithelial cell line (23). Further, these investigators showed that the proximal NF-κB element at position −115 bp of the human iNOS promoter was not required for cytokine induction of the promoter in the human epithelial cell lines AKN-1 and A549 (20), but others suggest that this element is important in A549 cells (22). Because cytokine induction in these cell lines was mediated by multiple NF-κB enhancer elements localized far upstream at −5.2 kb and beyond, it was suggested that species-specific differences exist in the transcriptional regulation of the iNOS gene (20). To add to the complexity, recent work in rat hepatocytes in primary culture revealed the functional importance of the proximal NF-κB site for cytokine induction of the human iNOS gene (39). Taken together, the available data highlight marked tissue-

specific differences in such transcription within the human species. In all likelihood, these differences evidence a diverse repertoire of regulatory control directed at the complex and multiple physiologic effects of the iNOS gene in various tissues and organs.

Our results also demonstrate the functional importance of C/EBP sites contained within the −0.2-kb region of the human iNOS promoter for both basal activity of the promoter and its activation by IL-1β. Indeed, deletion of all four C/EBP sites as well as the proximal NF-κB site suppressed the IL-1β inducibility of the iNOS promoter by more than 90% (Figure 8). Further, LIP, a dominant-negative C/EBPβ isoform, down-regulated markedly the basal level of promoter activity as well as its induction by IL-1β; by contrast, LAP, another C/EBPβ isoform that acts as a strong activator of transcription, up-regulated strongly the basal activity of the iNOS promoter essentially replicating the IL-1β-mediated induction (Figure 9). However, these data did not allow us to determine the precise contribution of each of the four C/EBP sites to the basal activity of the human iNOS promoter and its activation by IL-1β.

Which members of the C/EBP family of transcription factors are involved in the transcriptional activation of the human iNOS promoter by IL-1β in PVEC? Of the six members of the C/EBP family described to date, we investigated the potential role of C/EBPα, β and δ—members whose full-length isoforms contain an activation domain. Of the remaining three members, C/EBPγ and CHOP, have no such domain. We found that cotransfection with expression vectors for C/EBPα or C/EBPδ strongly activated the human iNOS promoter mimicking its induction by IL-1β, whereas overexpression of C/EBPβ was less effective (Figure 10). The results of Western blot analysis demonstrated, however, that C/EBPβ was the only member present in untreated PVEC, C/EBPα, δ, and ε being undetectable (Figure 11).

Overexpression of C/EBPβ in COS-1 cells or PVEC gave rise to at least four isoforms: the large-sized isoforms, LAP* and LAP, which feature activation domains and serve as transactivators; and the truncated isoforms, LIP and 14-kDa isoform, which retain the binding and dimerization domains but lack activation domains and serve as negative regulators. By contrast, only three isoforms were identifiable in untransfected and untreated PVEC: an isoform with molecular mass intermediate between LAP* and LAP, and bands corresponding to isoforms LAP and LIP. All three isoforms responded to IL-1β treatment by a similar level of up-regulation, the ratio between large-sized isoforms and truncated isoform remaining essentially unchanged (Figure 11A). Because this ratio determines the aggregate activity of C/EBPβ as a net activator or net repressor (28), we conclude that C/EBPβ is unlikely to be a major transactivator of the human iNOS promoter in response to IL-1β treatment.

Despite the presence of significant concentrations of the C/EBPβ isoforms LAP and LIP in untreated PVEC, as determined by Western blot analysis (Figure 11A), no DNA-protein complex appeared by EMSA using extracts from untreated PVEC and an oligonucleotide probe corresponding to C/EBP site #4 of the iNOS promoter (Table 1 and Figure 12). On the other hand, our supershift assay provided strong support for avid binding of C/EBPβ isoform contained in extracts from PVEC that had been treated with IL-1β to the C/EBP site of the iNOS promoter (Figure 12). This disparity might suggest that C/EBPβ undergoes some form of modification (e.g., phosphorylation) after IL-1β treatment that promotes binding to its cognate sequence. Considerable evidence indicates that phosphorylation of a number of residues at various domains of C/EBP results in activation of the factor (40). Additional data suggest that phosphorylation of C/EBPβ is essential for its translocation into the nucleus (41,42). Although phosphorylation of C/EBPβ can occur as a result of overexpression of p21ras (43) or treatment with a number of agents, including phorbol, 12-myristate 13-acetate (PMA) (44), the antioxidant pyrrolidine dithiocarbamate (PDTC) (42), calcium (45), and nerve growth factor (46), no direct evidence exists that such phosphorylation can result following treatment with IL-1β. Nonetheless, C/EBPβ overexpressed in COS-1 cells did not require IL-1β treatment for binding to C/EBP oligonucleotide in EMSA; however, the concentration of C/EBPβ in extracts from COS-1 cells was approximately 100-fold higher than those from PVEC.

Alternatively, these disparate results might reflect the inability of C/EBPβ to bind to C/EBP oligonucleotide as a homodimer. Induction of the C/EBPδ isoform after treatment with IL-1β (Figure 11B) might then allow formation of heterodimers with C/EBPβ and binding to DNA. Indeed, C/EBPβ and δ heterodimers have been shown to be better transactivators than C/EBPβ or δ homodimers (47). Nonetheless, overexpression of C/EBPβ in COS-1 cells, which have no endogenous C/EBP proteins, must have produced only C/EBPβ homodimers that were able to bind to DNA.

Although the C/EBPδ isoform was not present in untreated PVEC, it was strongly induced after IL-1β treatment (Figure 11B). The resulting band was similarly positioned to that obtained when C/EBPδ was overexpressed in COS-1 cells and corresponded to full-length C/EBPδ. No bands of lower molecular mass (analogous to the truncated isoforms of C/EBPβ) were obtained in either PVEC treated with IL-1β or COS-1 cells transfected with C/EBPδ expression plasmid. Thus, in contrast with C/EBPβ, in which IL-1β treatment of PVEC induced expression of both activator and repressor isoforms, such treatment induced only full-length C/EBPδ isoform thereby causing robust up-regulation of iNOS gene expression.

In accord with our findings, C/EBP site #4 (Table 1) was shown to be important for the induction of the human iNOS promoter by IL-1β in rat hepatocytes in primary culture, and C/EBPβ, but not C/EBPα or δ, was the isoform involved (39). Similarly, a critical role for C/EBP sites has been demonstrated in the activation of the rat iNOS promoter by LPS in rat neonatal cardiomyocytes (17) and by cAMP in rat mesangial cells (16). Further, activation of the murine iNOS promoter by LPS and INFγ in the mouse cell line ST-1 (medullary thick ascending limb of Henle's loop) required a C/EBP site (at position -150 to -142 bp) and binding of isoform C/EBPβ, but not C/EBPα, δ, or ε, was necessary (48).

It is not surprising that iNOS, a proinflammatory/immune response gene, is regulated by transcription factors NF-κB and C/EBP. Indeed, this is the case for a number of other genes involved in inflammatory and immune responses, including IL-6 (49), IL-8 (50), granulocyte-colony-stimulating factor (51), serum amyloid A protein (52), and cyclooxygenase-2 (53). Adding to the themes of complexity and tissue specificity that characterize the regulation of iNOS gene expression, no evidence of synergism between NF-κB and C/EBPβ was noted in PVEC, whereas such had been described in rat hepatocytes in primary culture (39). This difference might reflect the observation that, contrary to hepatocytes, activation of the human iNOS promoter in PVEC appears to involve C/EBPδ to a much larger extent than C/EBPβ. The prevailing levels of C/EBP isoforms and NF-κB in untreated cells might also contribute to differences in the regulation of the human iNOS gene in these two cell types. In our study, both C/EBP and NF-κB proteins were detectable by EMSA only after IL-1β treatment; by contrast, high levels of these factors were reported in untreated rat hepatocytes. These differences might be accounted for by variable activation of the regulatory cascades of these transcription factors in the two cell types. Indeed, the tissue-specific regulatory control that characterizes the iNOS gene might be exploited in the future for development of targeted preventive or therapeutic interventions.

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