ESE-1 Is a Novel Transcriptional Mediator of Inflammation That Interacts with NF-κB to Regulate the Inducible Nitric-oxide Synthase Gene*

Inflammation is a hallmark of several vascular diseases. The nuclear factor κB (NF-κB) transcription factors are dimeric proteins involved in the activation of a large number of genes in response to inflammatory stimuli. We report the involvement of a novel member of the ETS transcription factor, ESE-1, in mediating vascular inflammation. ESE-1 is induced in response to inflammatory cytokines and lipopolysaccharide in vascular smooth muscle cells, endothelial cells, and cells of the monocyte-macrophage lineage. This induction occurs within hours of stimulation and is mediated by NF-κB transactivation of the ESE-1 promoter. We have identified the inducible form of nitric-oxide synthase (NOS2) as a putative target for ESE-1. ESE-1 can bind to the p50 subunit of NF-κB, and cotransfection of ESE-1 with the p50 and p65 subunits of NF-κB synergistically enhances transactivation of the NOS2 promoter by ESE-1. An ESE-1-binding site within the NOS2 promoter has been identified, the site-directed mutagenesis of which completely abolishes the ability of ESE-1 to transactivate the NOS2 promoter. Finally, in a mouse model of endotoxemia, associated with acute vascular inflammation, ESE-1 is strongly expressed in vascular endothelium and smooth muscle cells. In summary, ESE-1 represents a novel mediator of vascular inflammation.

Inflammation is a prominent feature of several vascular diseases. The most common vascular disease, atherosclerosis, begins when lipoproteins, and in particular low density lipoprotein, enter the subendothelium and become oxidized. Oxidized low density lipoprotein stimulates the production of interleukin-1 and other inflammatory cytokines. These cytokines activate adhesion molecules, including VCAM-1, ICAM-1, and E-selectin, on the endothelial surface, which promote the attachment of and transmigration of monocytes. The expression of the inducible form of nitric-oxide synthase (NOS2) has also been shown to be up-regulated by inflammatory cytokines and endotoxin in cultured cells found in the atherosclerotic plaque including macrophages, smooth muscle cells, and endothelial cells (1–3). Furthermore, immunohistological studies have demonstrated the expression of NOS2 in the atherosclerotic lesions in these cell types as well (1, 4). The induction of the NOS2 is also associated with more acute forms of vascular inflammation such as endotoxemia. The generation of the potent vasodilator nitric oxide by NOS2 is at least in part responsible for the hypotension seen in association with bacterial sepsis (5, 6). NOS2 expression is also induced in other types of vascular inflammation including restenosis and in the accelerated atherosclerosis associated with heart transplantation (7, 8).

Upon binding of cytokines or other inflammatory mediators to their corresponding receptors, several classes of transcription factors are involved in the induction of these stimuli. For example, within minutes of interleukin-1β (IL-1β) treatment, the expression of the immediate early genes c-fos and c-jun are induced. These transcription factors are the constituent proteins for AP-1 (9, 10). One of the target genes of IL-1β, the collagenase gene, can be activated by AP-1 alone (11). Multiple signaling pathways have been implicated in the activation of these immediate early genes by IL-1β including the Janus kinases, mitogen-activated protein kinases, and protein kinase A (12–16).

The propagation of inflammation is dependent on several other transcription factors for the activation of multiple genes. The Rel/NF-κB transcription factor family is another set of genes, members of which are critical mediators of gene expression during inflammation. Activation of these factors does not require protein synthesis, as these factors are sequestered in the cytoplasm bound noncovalently to IκB-proteins, their endogenous inhibitors. Upon stimulation by inflammatory cytokines or endotoxin, these inhibitors are proteolytically degraded, allowing NF-κB to be translocated to the nucleus where it binds to the regulatory regions of target genes as a heterodimer. Although originally described as being important in lymphoid cells and lymphoid-specific genes, NF-κB has clearly been shown to play an important role in a whole host of other cell types and target genes. The p50 and p65 subunits of NF-κB bind to other transcription factors through protein interactions often resulting in synergistic transactivation of the target genes of NF-κB (17, 18).

The ETS genes are a family of at least 30 members that

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function as transcription factors (19). All ETS factors share a highly conserved 80–90-amino acid DNA binding domain, the Ets domain. ETS factors play a central role in regulating genes involved in development, cellular differentiation, and proliferation. Many macrophage-, B-, and T-cell-specific genes are regulated by ETS factors. The role of ETS factors in the immune system has been substantiated by experiments in mice where the genes encoding several ETS factors have been disrupted by homologous recombination. The PU.1 knockout is characterized by a lack of immune system development (20). T-cell apoptosis and increased terminal B-cell differentiation are characteristics of the Ets-1 knockout mice (21).

We recently identified a novel member of the ETS factor ESE-1 that is the prototype member of a new subclass of ETS factors (22). ESE-1 has several interesting features when compared with other ETS family members. First, unlike other ETS factors that are either ubiquitously expressed or primarily expressed in lymphoid cells, ESE-1 appears to have an epithelial specific expression pattern under basal conditions. Second, unlike all other ETS factors, ESE-1 has two DNA binding domains, a classical ETS domain and, in addition, an A/T hook domain also found in HMG proteins. In this report we demonstrate that ESE-1 is inducible in vascular smooth muscle cells, endothelial cells, and the cells of the monocyte-macrophage lineage, in response to inflammatory stimuli. This induction appears to be mediated via NF-kB. ESE-1 is able to bind directly to the p50 subunit of NF-kB and can augment the NF-kB-mediated activation of genes that are induced during inflammation. Finally, we have identified the NOS2 gene as a target for ESE-1 and have demonstrated the induction of ESE-1 in the mouse aorta in vivo during acute inflammation.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary human umbilical vein endothelial cells and primary human smooth muscle cells were obtained from Clonetics and grown according to the manufacturer’s recommendations. THP-1 and RAW 264.7 (monocytic cell lines) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Rat aortic smooth muscle cells (RASMCs) were harvested from male Harlan Sprague-Dawley rats by enzymatic dissociation according to the method of Gunther et al. (23).

RNA Isolation and Northern Blot Analysis—Total RNA was isolated using the RNAeasy kit (Qiagen). Northern blots were hybridized with random prime-labeled ESE-1 and glyceraldehyde-3-phosphate dehydrogenase cDNA in QuickHyb solution (Stratagene) according to the manufacturer’s recommendations and were washed at 50 °C with 0.2× SSC, 0.2% SDS.

RT-PCR Analysis—cDNAs were generated from 1 µg of mRNA isolated from different cells or tissues using oligo(dT)12–18 priming (Life Technologies, Inc.) and Moloney murine leukemia virus-reverse transcriptase (Life Technologies, Inc.) in deoxyribonuclease I (Life Technologies, Inc.)-treated samples. Each PCR used equivalent amounts of 0.1 ng of cDNA, 4 ng/µl of each primer, 0.25 units of Taq polymerase (Promega, Madison, WI), 150 µM of each dNTP, 3 mM of MgCl2, reaction buffer, and water to a final volume of 25 µl and were covered with mineral oil.

The sequences of the ESE-1 primers are as follows: sense, 5′-CTGAGCAAGAGTACTGGGACTGTC-3′, and antisense, 5′-CCATATGGTTGGCCACACGCTCAGG-3′, with an expected amplification product of 188 bp.

The sequences of the primers for glyceraldehyde-3-phosphate dehydrogenase are as follows: sense, 5′-CAAATGGTCTACATGAGGCT-3′, and antisense, 5′-CCATGGAGAAGGTCGCGG-3′, with an expected amplification product of 200 bp.

RT-PCR amplifications were carried out using a PerkinElmer Life Sciences thermal cycler 480 as follows: 20–30 cycles of 1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C followed by 15 min at 72 °C. Lower numbers of cycles were used to verify linearity of the amplification signal. 10 µl of the amplification product was analyzed on a 2% agarose gel.

In Vitro Transcription/Translation—Full-length cDNA encoding the whole open reading frame of p50 and p65 were inserted downstream of the T7 promoter into the pCRII TA cloning vector (Invitrogen). Coupled in vitro transcription/translation reactions were performed as described previously (24).

Electrophoretic Mobility Shift Assays—These were performed as described previously (25). In brief, 2 µl of in vitro translation product and 0.02–0.2 ng of 32p-labeled double-stranded oligonucleotides (5,000 cpm in 100 µl of 0.2× SSC) containing ETS sites (1 and 10 ng) were run on 4% polyacrylamide gels containing as buffer 0.5× TGE as described.

Glycerol-based oligonucleotides used as probes and for competition studies are as follows: 1) murine PSP promoter wild type oligonucleotide, 5′-TCGACGAAGATCCAGGAATAGGCT-3′ and 3′-GCTGGATGCGTTCC-TATCCCGAGGCT-5′; 2) HPRT promoter wild type ETS sites, 5′-GCGGAAAGAAGATGGGACTC-3′ and 3′-CCATCTCTCTGGTGAGG-5′.

Expression Vector and Luciferase Reporter Gene Constructs—A 1516- and 265-bp fragment corresponding to nucleotides −1485 to +31 and −234 to +31 of the murine NOS2 gene promoter were subcloned into the PGL2 luciferase reporter (Promega) (26).

Site-directed Mutagenesis—Site-directed mutagenesis of the NOS2 promoter was performed using the QuikChange Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer’s recommendations. In brief, PCR primers encoding the NOS2 promoter ETS site, −190 to −180, and flanking sequences, with TTAA substituted for GGAA were used. PCR was performed with Turbo polymerase using the wild type NOS2 promoter luciferase reporter construct as a template. The PCR reaction was digested with Dpn I and the undigested plasmids were transformed into DH5α bacteria. Individual minipreps were sequenced to verify incorporation of the ETS site mutation.

DNA Transfection Assays—Cotransfections of 2 × 105 RAW cells or RASMCs were carried out with 0.6 µg of reporter gene construct DNA and 0.6 µg of expression vector DNA using 6 µl of LipofectAMINE (Life Technologies, Inc.) as described (27). The cells were harvested 16 h after transfection and assayed for luciferase activity. Transfections for every construct were performed independently in duplicates and repeated 3 times with two different plasmid preparations with similar results. Cotransfection of a second plasmid for determination of transfection efficiency was omitted because potential artifacts with this technique have been reported and because many commonly used viral promoters contain potential binding sites for ETS factors (28).

GST Pull-down Assay—A series of GST-ESE-1 fusion proteins were generated by PCR with specific primers to contain in frame restriction enzyme sites and sequenced to confirm that there were no mutations introduced by the PCR. GST-ESE-1 fusion proteins were prepared as described previously (29). [35S]Methionine-labeled and in vitro translated full-length p50 and p65 were incubated with equal amounts of GST, GST-ESE-1 fusion protein, and agarose beads in 200 µl of NETN (0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 100 mM NaCl) for 3 h at 4 °C with gentle shaking. Bound p50 or p65 proteins were then eluted after three washings with NETN buffer and analyzed on a 12% SDS-polyacrylamide gel.

Rodent Model of Endotoxemia—The model used is as described previously (30). In brief, C57Bl/6 mice were injected with Salmonella typhosa LPS (20 mg/kg intraperitoneally). Aortas were harvested 4 h after injection, fixed, and stained for ESE-1 protein expression as described below.

Immunohistochemistry—Using the models described above, the mice were euthanized after 4 h of LPS treatment, and the blood vessels were perfusion-fixed with 4% paraformaldehyde via the left ventricle. Following fixation, the blood vessels were paraffin-embedded. Deparaffinized 5-µm sections were incubated with a rabbit polyclonal anti-ESE-1 antibody. The primary antibody was applied at a concentration of 1/200 for 1 h at room temperature and then left overnight at 4 °C. After washing, sections were incubated with a biotinylated goat anti-rabbit antibody (Vectastain ABC, Vector Labs, Burlingame, CA) at 1.5 µg/ml in phosphate-buffered saline, 0.4% Triton X-100 for 1 h at room temperature. After they were incubated with avidin, the sections were washed, incubated with a biotinylated goat anti-rabbit antibody (Vectastain ABC, Vector Labs, Burlingame, CA) at 1.5 µg/ml in phosphate-buffered saline, 0.4% Triton X-100 for 1 h at room temperature. After they were incubated with avidin, the sections were washed and incubated with a peroxidase 3,3′-diaminobenzidine kit (Vector Laboratories). The ESE-1 antibody was generated in our laboratory, and the NOS2 antibody was obtained from Santa Cruz Biotechnology.

RESULTS

ESE-1 Is Inducible in Response to Inflammatory Stimuli—We have previously shown that under basal conditions ESE-1 expression is restricted to cells of epithelial origin (22). To examine whether ESE-1 could be induced in nonepithelial cells, ESE-1 expression was evaluated in human aortic smooth muscle cells, human umbilical endothelial cells, and the human monocyte-macrophage cell line (THP-1). As shown in Fig. 1A,
ESE-1 expression is induced in response to the inflammatory cytokines interleukin-1β and TNF-α and endotoxin. Interestingly, depending on the cell type, ESE-1 expression was induced as early as 1 h in the THP-1 cells to as late as 4 h in the human aortic smooth muscle cells. In all cells tested ESE-1 expression was almost completely absent by 24 h after stimulation, suggesting that ESE-1 expression in these cell types is strongly linked to stimulation by inflammatory stimuli. To confirm the results obtained by RT-PCR, we performed Northern blot analysis of ESE-1 using total RNA derived from human aortic smooth muscle cells stimulated with interleukin-1β. As shown in Fig. 1B, ESE-1 expression is absent prior to induction with IL-1β, and expression peaks about 4 h after stimulation.

The ESE-1 Promoter Is Inducible in Response to Inflammatory Cytokines—We have previously isolated and characterized the human ESE-1 promoter (31). As shown in Fig. 2A, the ESE-1 promoter contains a putative NF-κB-binding site, in addition to sites for other known transcription factors such as AP-1, ETS, and CRE. To test whether the ESE-1 promoter was responsive to an inflammatory stimulus, a luciferase reporter construct containing the ESE-1 promoter was transfected into the mouse monocytic RAW 264.7 cell line. As shown in Fig. 2B, the ESE-1 promoter is inducible to LPS in the RAW cells, and a mutation in the NF-κB site significantly reduces the inducibility suggesting that binding of NF-κB or related Rel proteins is required for inducible ESE-1 expression (Fig. 2C). The same results were obtained in the rat aortic smooth muscle cells (data not shown).

ESE-1 Induction Is NF-κB-mediated—To identify the specific proteins binding to the ESE-1 NF-κB site, EMSAs were performed using whole cell extracts derived from human aortic smooth muscle cells stimulated with IL-1β. As shown in Fig. 3A, IL-1β stimulation is associated with an inducible change in binding pattern to the ESE-1 NF-κB-binding site. Interestingly, this binding occurs as early as 1 h, despite the fact that ESE-1 expression was only detected in IL-1β-stimulated HASMCs at about 4 h. To identify the proteins specifically binding to this site, EMSAs were performed in the presence or absence of antisera to the different Rel family members. As shown in Fig. 3B, the only two proteins recognized in this complex are p50 and p65. This suggests that the induction of ESE-1 is mediated by inducible binding of the dimeric NF-κB proteins p50 and p65 to the ESE-1 promoter.

**NOS2 Is a Potential Target Gene for ESE-1**—In an attempt to identify target genes for ESE-1, the ability of ESE-1 to transactivate the promoters of several genes that are induced in response to inflammatory stimuli were tested. As shown in Fig. 4A, ESE-1 significantly transactivates the NOS2 promoter (5-fold) but not E-selectin, CD44, or the interleukin-6 promoters. Transactivation of the ICAM-2 promoter by ESE-1 led to a 3.5-fold induction. The promoter of another gene, Fli-1, which is known to be regulated by ETS factors, Flt-1, but is not induced in response to inflammatory cytokines was used as another control was mildly induced by ESE-1. The NOS2 gene promoter has two important regulatory regions, one that is upstream (−1100 to −800) and one that is

![Fig. 1. ESE-1 induction to inflammatory stimuli.](url)

![Fig. 2. ESE-1 promoter and induction by LPS.](url)
in the downstream region proximal to the transcription start site (−234 to +31). To test whether ESE-1 transactivation occurred principally through the upstream region or through the region near the transcription start site, cotransfections were performed with ESE-1 and reporter constructs encoding either a long fragment of the NOS2 promoter containing both regions or one containing just the downstream promoter region. As shown in Fig. 4B, transactivation by ESE-1 was similar with both NOS2 promoter constructs tested, suggesting that most of transactivation by ESE-1 occurs through the promoter region near the transcription start site (−234 to +31).

ESE-1 Can Bind to an ETS Site within the NOS2 Promoter—By having identified putative targets for ESE-1, we were also interested to determine whether ESE-1 could specifically bind to ETS sites within the NOS2 promoter. An ETS-binding site was identified that is highly homologous to the ETS consensus binding site for ESE-1 (33). By using in vitro translated ESE-1 protein, EMSA was performed comparing the potential binding of ESE-1 to the NOS2 ETS site with an ETS site in the epithelial specific PSP gene previously shown to bind to ESE-1 (33). As shown in Fig. 5, ESE-1 binds equally well to both ETS sites.

Mutational Analysis of the NOS2 Promoter ETS Site—To determine the functional importance of the NOS2 promoter ESE-1-binding site (−190 to −180), site-directed mutagenesis of this site, substituting “TTAA” for the core “GGAA” sequence, was performed. Two independent constructs were verified by DNA sequencing. The effect of this mutation upon the ability of ESE-1 to transactivate the mutated NOS2 promoter was first examined. As shown in Fig. 6A, this mutation completely abolished the ability of ESE-1 to transactivate the NOS2 promoter. The functional importance of this mutation was next evaluated in the context of induction with an inflammatory stimulus. As shown in Fig. 6B, the same mutation led to a 60% reduction in inducibility of the promoter with LPS. This reduction is similar to that seen with mutation of the NF-κB or OCT sites (34).

ESE-1 Synergizes with p50 and p65 to Transactivate the NOS2 Promoter—Because p50 and p65 are also activated in response to inflammatory stimuli, we were interested to examine whether the interaction of the Rel domain proteins with ESE-1 has any functional effect upon transactivation. Cotransfection experiments with ESE-1, p50, and p65 were performed with the NOS2 promoter in RAW264.7 and rat aortic smooth muscle cells. As shown in Fig. 7, although both ESE-1 and the combination of p50 and p65 were able to transactivate the NOS2 promoter, there was a marked synergistic response with the combination of p50, p65, and ESE-1 in both cell types tested. Thus, ESE-1 substantially augments the NF-κB-mediated transcriptional response to inflammatory stimuli.

ESE-1 Binding to Rel Domain Proteins—It has previously been shown that both ETS factors and HMG proteins are capable of binding to the Rel family members (35, 36). Interestingly, the binding regions where these protein-protein interactions occur are within the DNA binding domain of both types of transcription factors. For the HMG(I)Y protein, this region of interaction has been precisely mapped within one of the AT hook domains (36). There is significant protein sequence homology between the AT hook domain of ESE-1 and the region responsible for binding with p50 in the HMG(I)Y protein. Although the ETS domain is known to interact with p50, the precise region within this domain has not been determined. To identify whether ESE-1 can bind to either the p50 or p65 Rel domain proteins, [35S]methionine-labeled p50 and p65 were generated by in vitro translation (See Fig. 8A). Several GST-
ESE-1 fusion constructs and the GST protein alone were made. A diagram of the constructs is shown in Fig. 8C. The GST fusion binding studies demonstrate that not only does the full-length ESE-1 protein bind to p50 but both the A/T hook and the ETS domain were capable of interacting with p50 (Fig. 8B). In contrast, there was no significant binding of any of the constructs to p65 (data not shown).

ESE-1 Is Induced in Acute Vascular Inflammation—To examine the vascular expression of ESE-1 in response to an inflammatory stimulus, ESE-1 expression was evaluated by immunohistochemical analysis in a rodent model of endotoxemia in which marked vascular inflammation occurs within hours of endotoxin administration. As shown in Fig. 9, ESE-1 expression was markedly induced 4 h after exposure to endotoxin. Intense expression of ESE-1 is shown in the vascular endothelium (see arrows) and first layer of the vascular smooth muscle cells. Some expression is also evident in some of the other layers of vascular smooth muscle cells. In contrast, minimal to no ESE-1 expression was observed in the mouse aorta at baseline. To determine whether ESE-1 has a similar expression pattern as NOS2 in this model of inflammation, we examined the same aortic specimens using a NOS2-specific antibody. As shown in Fig. 9, NOS2 is expressed in the endothelium and first layer of vascular smooth muscle cells, similar to ESE-1 expression. These studies confirm the inducibility of ESE-1 in response to inflammatory stimuli, in vivo as well as in vitro, and further support the role of ESE-1 in regulating NOS2 expression.

**DISCUSSION**

The ETS transcription factor family has been shown to regulate a wide variety of normal cellular responses, including cellular growth and differentiation. The promoters of several genes that are induced in response to inflammatory cytokines have conserved binding sites for ETS factors that are functionally important. The tumor necrosis factor-α (TNF-α) gene promoter contains ETS-binding sites that are functionally important for inducibility by phorbol esters (37). Induction of JunB by interleukin-6 has been demonstrated to require binding to a
conserved ETS site (38). The induction of both the macrophage inflammatory protein-1 (MIP-1α) and the chemokine regulated on activation normal T-cell expressed by inflammatory cytokines has been shown to be at least in part mediated by ETS factors (39, 40). The specific ETS factors responsible for the regulation of these genes has not been identified. The effect of inflammatory cytokines on the expression of selected ETS factors has been variable. Macrophage gene expression of the ETS factor Fli-1 is reduced in response to LPS, IL-1β, or phorbol 12-myristate 13-acetate (41). In contrast, Ets-1 expression could be increased in response to phorbol 12-myristate 13-acetate and platelet-derived growth factor in rat vascular smooth muscle cells (42). The proposed targets for Ets-1 include the matrix metalloproteinases collagenase and stromelysin, which contain ETS-binding sites in their respective promoters. TNF-α has also been shown to induce Ets-1 in vascular smooth muscle cells (43). Finally, Ets-1 has also been shown to be induced in fibroblasts in response to stimulation with basic fibroblast growth factor, epidermal growth factor, and platelet-derived growth factor (44). Although Ets-1 expression can be increased in response to a variety of stimuli in these cell types, Ets-1 is already expressed at base line in these cells. In contrast, the expression pattern of ESE-1 is quite different at base line in that it is predominantly expressed in cells of epithelial origin and completely absent in vascular smooth muscle cells, endothelial cells, or monocytes. The rapid and transient induction of ESE-1 suggests that the target genes for ESE-1 in these cell types are more likely to be directly associated with mediating the inflammatory response than for the other ETS transcription factors that are already expressed at base line.

NOS2 is also induced in response to inflammatory cytokines in vascular smooth muscle cells, endothelial cells, and monocytes. ETS factors have not previously been shown to be important for the inducibility of the NOS2 gene. However, it has recently been determined that the expression of the NOS1, which is predominantly found in neurons and has generally been thought to be constitutively expressed, can be up-regulated with nerve growth factor. Analysis of the NOS1 promoter demonstrates multiple potential ETS-binding sites in the regulatory region responsible for induction; however, no specific ETS factors have been identified that regulate this gene (45). Interestingly, we have also recently determined that ESE-1 is inducible in other nonepithelial cells in response to inflammatory cytokines, including glial cells. Although NOS2 is generally considered an inducible enzyme, which is not constitutively expressed, NOS2 is highly expressed in fetal and adult bronchial epithelium (46). Interestingly, we also determined the highest level of ESE-1 expression in bronchial epithelium (22). The functions of nitric oxide in the mature airways include smooth muscle relaxation, neurotransmission, bacteriostasis, and modulation of plasma exudation, mucin secretion, and ciliary motility. Constitutive expression of NOS2 has also recently been demonstrated at lower levels in both gastric and colonic epithelium and is enhanced in association with infection or neoplasia (47, 48).

The induction of the murine NOS2 by endotoxin, IL-1β, and interferon requires binding of specific transcription factors to regulatory regions within the NOS2 promoter. An NF-κB site (−85 to −76) is critical for activation of NOS2 by LPS and inflammatory cytokines in macrophages and vascular smooth muscle cells (49, 50). An upstream promoter/enhancer region (−951 to −911) is responsible for the synergistic activation of NOS2 by interferon-γ and LPS. The specific transcription fac-

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**FIG. 7.** Synergistic effect of NF-κB with ESE-1 upon NOS2 transactivation. Cotransfection experiments of different combinations of the pCI mammalian expression plasmid containing cDNAs encoding either ESE-1, p50, or p65, were performed in RAW 264.7 (A) or RASMCs (B), with the NOS2 promoter luciferase reporter construct (short).

**FIG. 8.** ESE-1 interacts with p50. A, [35S]methionine in vitro translated rabbit reticulocyte lysates of p50, p65, and unprogrammed lysate (control) separated by SDS-gel electrophoresis. Molecular weight markers are shown on the left. B, binding of p50 to ESE-1, another ETS factor NERF-2, and several deletion constructs, and the GST fusion protein alone (control). C, schematic of ESE-1 constructs used in GST fusion experiments. (See “Experimental Procedures” for details of the GST pull down experiment.)
tors binding to this region include interferon regulatory factor-1, and Stat1a (51, 52). The transactivation of the proximal promoter (~234 to +31) in response to LPS or interleukin-1β can partially be blocked by mutating the NF-κB site (34). Inducible binding of additional factors to other sites within this region has been demonstrated by in vivo footprinting experiments. These sites include an NF-IL6 (~150 to ~142) site and an octamer (~61 to ~54) site (53). Mutational analysis of the OCT site has demonstrated the functional importance of this site (34). Furthermore, the transcription factor HMG-I(Y) has been shown to bind to the OCT site and cooperatively interact with NF-κB to form a ternary complex and potentiate NOS2 transcription (34). Our results are the first demonstration that Ets factors may be able to regulate NOS2 expression.

Unlike any other ETS factors, ESE-1 is unique in that it has an A/T hook DNA binding domain in addition to the classical ETS domain. A/T hook domains are found in another family of transcription factors, the high mobility group (HMG) proteins. These factors are nonhistone chromosomal proteins that alter chromatin structure by binding to A/T-rich DNA sequences (54). DNA binding of HMG proteins is mediated by A/T hook DNA binding domains that often exist in tandem clusters of two or three domains that facilitate stronger binding to two or more tandemly placed A/T-rich DNA sequences. HMG proteins have recently been implicated in enhancing inflammatory responses (30). In addition to affecting DNA structure, they also act to enhance transcription by recruiting additional proteins such as NF-κB, ATF-2/c-Jun, and interferon regulatory factor-1 (34). The binding of the p50 subunit of NF-κB has been shown to be mediated via one of these A/T hook domains (36). HMG-I(Y) expression is induced in vascular smooth muscle cells in response to inflammatory stimuli including endotoxin and interleukin-1β (30). The A/T hook domain within ESE-1 may facilitate mediation of the inflammatory response either by recruiting other proteins such as p50 or by enhancing binding of ESE-1 or other factors by altering DNA structure through binding to A/T-rich sequences.

The ability of other ETS factors to bind to the p50 subunit of NF-κB has been shown for other Ets factors including Ets-1 and ELF-1 (32, 35). For both of these Ets factors the interaction occurs via the conserved Ets DNA binding domain. It is therefore not surprising that the Ets domain of ESE-1 is one of the domains capable of interacting with p50. The degree of synergism associated with cotransfection of Ets factors with the p50 and p65 subunits of NF-κB upon transactivation of the regulatory regions of different genes is variable. For example, when the enhancer regions of the HIV-1 and HIV-2 genes, which are known to bind to Ets factors, were tested, there was a maximal 4–5-fold total transactivation when cotransfection experiments were performed with the combination of Ets-1, p50, and p65 (35). When the same type of experiment was performed with the GM-CSF promoter, this led to a 100-fold activation of this promoter, which was ~10-fold greater than with either Ets-1 or NF-κB alone (32). Thus, the functional role of these interactions may be highly dependent upon the particular target genes. For expression of NOS2, there was a marked synergistic effect with the combination ESE-1, p50, and p65.

In summary, our study demonstrates that ESE-1 is a novel ETS factor that is induced in response to inflammatory stimuli and augments NF-κB-mediated induction of gene targets associated with inflammation. In particular, we have identified the inducible isofrom of nitric-oxide synthase (NOS2) as a target for ESE-1.

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Fig. 9. Expression of ESE-1 in the mouse aorta during acute inflammation. Immunohistochemical evaluation of ESE-1 protein expression in the mouse aorta before and 4 h after systemic administration of endotoxin (see “Experimental Procedures” for details). Control represents immunohistochemical evaluation with ESE-1 antibody at base line prior to administration of endotoxin. NOS2 staining is performed at the 4-h time point.
ESE-1 Is a Novel Transcriptional Mediator of Inflammation That Interacts with NF-κB to Regulate the Inducible Nitric-oxide Synthase Gene

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