Elk-3 Is a Transcriptional Repressor of Nitric-oxide Synthase 2*

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The inducible isoform of nitric-oxide synthase (NOS2), a key enzyme catalyzing the dramatic increase in nitric oxide by lipopolysaccharide (LPS), plays an important role in the pathophysiology of endotoxia and sepsis. Recent evidence suggests that Ets transcription factors may contribute to NOS2 induction by inflammatory stimuli. In this study, we investigated the role of Ets transcription factors in the regulation of NOS2 by LPS and transforming growth factor (TGF)-β1. Transient transfection assays in macrophages showed that Ets-2 produced an increase in NOS2 promoter activity, whereas the induction by Ets-1 was modest and NERF2 had no effect. Elk-3 (Net/Erp/Sap-2a) markedly repressed NOS2 promoter activity in a dose-dependent fashion, and overexpression of Elk-3 blunted the induction of endogenous NOS2 message. Mutation of the Net inhibitory domain of Elk-3, but not the C-terminal-binding protein interaction domain, partially alleviated this inhibitory domain of Elk-3, but not the C-terminal-binding domain; TCF, ternary complex factors; IFN, interferon.

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Sepsis is a systemic inflammatory response to severe infection that frequently progresses to refractory hypotension, multiple organ system failure, and death (1). In a subset of patients, the release of bacterial cell wall-derived lipopolysaccharide (LPS) or endotoxin initiates a cascade of inflammatory events (2). This response to infection is mediated, in part, by macrophage activation and the release of proinflammatory cytokines (3). Among the mediators induced by these signal cascades, nitric oxide (NO) acts as a key regulator of hypotension and end organ damage (4, 5). The production of nitric oxide from L-arginine is catalyzed by NO synthases (NOS) (6). Of the three isoforms of NOS identified in mammalian cells, the expression of NOS2 is inducible by many proinflammatory stimuli, such as interleukin-1β, tumor necrosis factor-α, interferon-γ, and bacterial LPS, depending on the target cell. Thus, the study of NOS2 gene regulation is of particular importance in our understanding and management of sepsis and other inflammatory disorders.

Ets proteins are a family of transcription factors that share a unique and highly conserved, winged helix-turn-helix DNA-binding domain (7). Ets proteins are involved in the transcriptional regulation of genes important for determining tissue specificity, cellular differentiation, and cellular proliferation by binding to GGA(A/T) sites in the promoters/enhancers of these genes (7–9). Several reports have demonstrated a vital role for Ets family proteins in the regulation of mammalian immunity (8, 10, 11). Additional reports have also revealed that Ets family proteins regulate gene expression in non-immune cell types. For example, Dube et al. (12) showed that the Ets transcription factor NERF2 is an important mediator of the vascular-specific expression of TIE2 in endothelial cells. In addition, ESE-1 represents a novel mediator of vascular inflammation (13). Interestingly, a target gene of ESE-1 in vascular cells and macrophages is NOS2 (13). Due to the potential importance of Ets factors in immune cells and vascular inflammation, we wanted to determine whether other members of the Ets superfamily could regulate NOS2 gene expression.

Beyond the induction of NOS2 by proinflammatory stimuli, previous work from our laboratory has focused on the role of transforming growth factor (TGF)-β1 in the down-regulation of NOS2 after its induction by proinflammatory cytokines and LPS (14–17). TGF-β1 inhibited NOS2 at the level of gene transcription (14), and this effect occurred in part by down-regulating the expression of high mobility group (HMG)-1/Y protein (17), an architectural transcription factor. However, we hypothesized that other mechanisms may exist for the inhibition of the NOS2 gene by TGF-β1. Among the Ets family members, a candidate gene known to have transcriptional repressive activity is Elk-3 (Net/Erp/Sap-2a) (18–22). Elk-3 is a member of the ternary complex factors (TCF) subfamily of Ets proteins, along with Elk-1 and Sap-1. TCFs have been shown to participate in the response of the cell to Ras and growth sig-

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§ The abbreviations used are: LPS, lipopolysaccharide; NO, nitric oxide; NOS, NO synthase(s); iNOS, inducible NOS; TGF-β1, transforming growth factor-β1; NID, Net inhibitory domain; CID, C-terminal-binding protein interaction domain; TCF, ternary complex factors; IFN, interferon.
sion vector (Invitrogen) at the NcoI sites. When compared with Elk-1 and Sap-1, Elk-3 is unique in that it exhibits strong transcription repressor activity under basal conditions. Prior studies have shown that this inhibitory effect occurs through the Net inhibitory domain (NID) (22) and the C-terminal-binding protein interaction domain (CID) (24). The present study was designed to investigate the role of Etas transcription factors in the regulation of NOS2 by LPS and TGF-β1 in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Murine macrophages (RAW 264.7) were cultured as described previously (25), and NIH3T3 cells were cultured according to recommendations from American Type Culture Collection. LPS from Escherichia coli (serotype O26:B6) was purchased from Sigma, and human recombinant TGF-β1 and interleukin-1β (IL-1β) and Sigma and recombinant interferon (IFN)-γ were purchased from PeproTech Inc. (Rocky Hill, NJ).

Plasmid Constructs and DNA Probes—The mouse NOS2 luciferase reporter plasmids, iNOS(−1485/+31), iNOS(−815/+31), and iNOS(−234/+31) in the pGL2-Basic vector (Promega) were described previously (14, 15). Ets-1, Ets-2, NERF2, and Elk-3 (Ets domain that mediates DNA binding), B (interaction with a repressive domain) and D (mitogen-activated protein (MAP) kinase docking domain) (22, 23). When compared with Elk-1 and Sap-1, Elk-3 is unique in that it exhibits strong transcription repressor activity under basal conditions. Prior studies have shown that this inhibitory effect occurs through the Net inhibitory domain (NID) (22) and the C-terminal-binding protein interaction domain (CID) (24).

To explore the role of Etas transcription factors in transfectional regulation of the NOS2 promoter in macrophages, we transfected RAW 264.7 cells with expression constructs for several Etas proteins (including Ets-1, Ets-2, NERF2, and Elk-3) and assessed promoter activity of the iNOS(−1485/+31) reporter construct. Using transient transfection assays in the absence of LPS, we found that Ets-2 stimulated NOS2 promoter activity (−3-fold induction, p < 0.05), whereas the Ets-1 induction was modest (<2-fold, not significant) and NERF2 had no effect (Fig. 1). After 24 h of treatment with LPS, NOS2 promoter activity with control empty plasmid (pCI) was induced <7-fold, and the addition of Ets-2 with LPS produced a 16-fold induction (p < 0.05). In the presence of LPS, Ets-1 induced NOS2 promoter activity 12-fold, even in the presence of LPS. Elk-3, in contrast, showed a strong repressive effect (80–85% at a concentration of 200 ng/well), not significant) and NERF2 had no effect (Fig. 1). After 24 h of treatment with LPS, NOS2 promoter activity with control empty plasmid (pCI) was induced <7-fold, and the addition of Ets-2 with LPS produced a 16-fold induction (p < 0.05). In the presence of LPS, Ets-1 induced NOS2 promoter activity 12-fold, although this was not significantly different from NOS2 promoter activity transfected with control plasmid (7-fold).

Statistics—Where indicated, comparisons between groups were made by factorial analysis of variance followed by Fisher’s least significant difference test.

RESULTS

Elk-3 Represses NOS2 Promoter Activity—To explore the role of Etas factors in transcriptional regulation of the NOS2 promoter in macrophages, we transfected RAW 264.7 cells with expression constructs for several Etas proteins (including Ets-1, Ets-2, NERF2, and Elk-3) and assessed promoter activity of the iNOS(−1485/+31) reporter construct. Using transient transfection assays in the absence of LPS, we found that Ets-2 stimulated NOS2 promoter activity (−3-fold induction, p < 0.05), whereas the Ets-1 induction was modest (<2-fold, not significant) and NERF2 had no effect (Fig. 1). After 24 h of treatment with LPS, NOS2 promoter activity with control empty plasmid (pCI) was induced <7-fold, and the addition of Ets-2 with LPS produced a 16-fold induction (p < 0.05). In the presence of LPS, Ets-1 induced NOS2 promoter activity 12-fold, although this was not significantly different from NOS2 promoter activity transfected with control plasmid (7-fold). NERF2 had no additional effect on NOS2 promoter activity, even in the presence of LPS. Elk-3, in contrast, showed a strong repressive effect (80–85% at a concentration of 200 ng/well, p < 0.05) on the promoter activity of iNOS(−1485/+31), in the presence or absence of LPS.

Elk-3 Represses NOS2 Promoter Activity in a Dose-dependent Manner—We next wanted to determine whether the effect of Elk-3 on LPS-mediated NOS2 induction was contingent on Elk-3 plasmid concentration. As shown in Fig. 2, the repressive effect of Elk-3 on the NOS2 promoter in the presence of LPS...
Elk-3 inhibition of NOS2

**Elk-3 represses NOS2 promoter activity in a dose-dependent fashion.** RAW 264.7 cells were transiently co-transfected with luciferase reporter plasmids (NOS2, Ets-2, Ets-1, or Elk-3) (10 ng/ml) for 24 h and then harvested. Luciferase activity was measured as indicated. The empty pCI vector was added to keep the total plasmid DNA concentration constant. After transfection, cells were treated with vehicle (−) or LPS (2 μg/ml) for 24 h and then harvested. Luciferase activity was plotted as a fold induction of NOS2 promoter activity from cells receiving no Elk-3 expression plasmid and no LPS. Three independent experiments were performed in duplicate. *, p < 0.05 versus control without LPS or Elk-3; †, p < 0.05 versus LPS-treated group without Elk-3 co-transfection.

Overexpression of Elk-3 Represses Endogenous NOS2 mRNA Levels—To determine whether overexpression of Elk-3 could regulate endogenous NOS2 expression, we performed transient transfection analyses of NIH/3T3 cells in the presence or absence of proinflammatory stimuli (LPS, IL-1β, and IFN-γ). NIH/3T3 cells were used because of their higher transfection efficiency when compared with RAW 264.7 cells. After transfection with the control empty plasmid (pCI) or Elk-3, we treated NIH/3T3 cells with LPS (2 μg/ml), IL-1β (10 ng/ml), and IFN-γ (100 units/ml) and then harvested total RNA after 24 h. Northern blot analysis revealed that when compared with control pCI plasmid, overexpression of Elk-3 repressed endogenous NOS2 mRNA that had been induced by LPS, IL-1β, and IFN-γ (Fig. 3).

Ets Binding Site in the Downstream NOS2 Promoter (−190 to −180) Contributes to Ets-1 and Ets-2, but Not Elk-3, Alteration in NOS2 Promoter Activity—A critical Ets binding site in the downstream NOS2 promoter (bp −190 to −180) is important for the regulation of NOS2 promoter activity by the Ets transcription factor ESE-1 (13). To evaluate the function of this Ets binding site on Ets-1, Ets-2, and Elk-3 regulation of the NOS2 promoter, we performed deletion analysis and site-directed mutagenesis of the NOS2 promoter. Using several different NOS2 deletion constructs, iNOS (−1345/+31), iNOS (−815/+31), iNOS (−518/+31), and iNOS (−234/+31), we found that all of the constructs responded similarly to Ets-2 and Ets-1 induction, or Elk-3 repression, in RAW 264.7 cells (data not shown). However, mutation of the downstream Ets site (bp −190 to −180) in the iNOS (−234/+31) construct led to a significant blunting of LPS-induced NOS2 promoter activity by Ets-2 and Ets-1 (p < 0.05), when compared with wild-type promoter activity (Fig. 4). In contrast, mutation of this Ets site had no effect on the ability of Elk-3 to repress NOS2 promoter activity that was induced by LPS.

**Elk-3 Is Down-regulated by LPS in Cultured Macrophages and Mouse Tissues**—To investigate the endogenous regulation of Elk-3 expression in the presence of LPS, we harvested total RNA from RAW 264.7 cells that were treated with increasing doses of LPS (from 0 to 100 ng/ml) for 6 h. By Northern blot analysis, we found that mRNA levels of Elk-3 decreased in a dose-dependent fashion as the LPS dose increased (Fig. 6A). Data from mRNA levels of three independent experiments showed that LPS significantly decreased Elk-3 mRNA levels after 6 h at a dose of 1 ng/ml (p < 0.05), and the expression of NOS2 mRNA began to increase at this same dose (Fig. 6B, p < 0.05). Maximal suppression of Elk-3 mRNA (94% decrease) occurred at an LPS dose of 100 ng/ml, whereas NOS2 mRNA was dramatically induced. Furthermore, we investigated the relationship between Elk-3 and NOS2 expression in mice during endotoxemia. Northern blot analysis showed that the expression of Elk-3 was strongly repressed in mouse kidney, lung, and heart tissues 4 h after the administration of LPS, whereas NOS2 was induced (Fig. 7). By 12 h after LPS administration, the mRNA levels of Elk-3 increased and the mRNA levels of NOS2 decreased, reverting back toward their respective baseline levels. Taken together, these data demonstrate that endogenous expression of Elk-3 inversely correlates with NOS2 expression after treatment with LPS.

**TGF-β1 Reverses the Suppression of Elk-3 by LPS and Decreases NOS2 Expression**—We have reported previously that TGF-β1 significantly down-regulates NOS2 promoter activity and mRNA levels that had been induced by proinflammatory cytokines. NIH/3T3 cells were transiently transfected with either an expression plasmid encoding Elk-3 (6 μg/well) or the corresponding empty vector (pCI, 6 μg/well). After transfection, the cells were stimulated with vehicle (−) or LPS (2 μg/ml), IL-1β (10 ng/ml), and IFN-γ (100 units/ml). Total RNA was extracted 24 h after vehicle or LPS/IL-1β/IFN-γ administration, and Northern blot analyses were performed as described under “Experimental Procedures,” using 32P-labeled NOS2, Elk-3, and 18S RNA probes. This experiment was performed two independent times.
cytokines or LPS in vascular smooth muscle cells and macrophages, respectively (14, 15, 17). Since endogenous Elk-3 expression inversely correlates with NOS2 message, and overexpression of Elk-3 represses the induction of NOS2 by an inflammatory stimulus, we wanted to determine whether TGF-β (10 ng/ml) would increase Elk-3 mRNA that had been down-regulated by LPS (10 ng/ml). Total RNA was harvested from RAW 264.7 cells at various time points after LPS administration, as shown in Fig. 8. Northern blot analysis revealed that after LPS administration, Elk-3 mRNA levels decreased rapidly (as early as 1 h, data not shown), with a maximal suppression by 6 h (Fig. 8). Moreover, this decrease in Elk-3 mRNA by LPS preceded the induction of NOS2. TGF-β significantly increased Elk-3 mRNA levels that had been down-regulated by LPS, and this increase in Elk-3 correlated with a TGF-β-induced down-regulation of NOS2. Since overexpression of Elk-3 can down-regulate LPS-induced NOS2 (Figs. 1–3), these data suggest that TGF-β may down-regulate NOS2, in part, by up-regulating Elk-3.

**DISCUSSION**

The inflammatory response is a key component of host defense, but excessive activation of the immune system and subsequent release of vasoactive mediators, as occurs in endotoxemia and sepsis, may be fatal (3). Overproduction of NO, through LPS and proinflammatory cytokine activation of NOS2, acts as a key regulator of hypotension and end organ damage (4, 5). Due to the detrimental consequences of an exaggerated response to infection, the immune system must be tightly controlled to prevent a massive systemic reaction leading to multiple organ failure and death. The effects of proinflammatory cytokines can be counterbalanced by the production of anti-inflammatory mediators (3). Thus, understanding the endogenous mediators responsible for the transcriptional regulation of NOS2 (either up-regulation or down-regulation) may provide important insight into the pathophysiology of systemic inflammatory processes such as endotoxemia and sepsis.

In collaboration with Rudders et al (13), we reported previously that ESE-1, a member of the immune modulatory family of Ets proteins, regulates NOS2 expression and is a mediator of vascular inflammation. In the present study, we extend these findings by investigating the role of additional Ets transcription factors in the regulation of NOS2 by LPS and TGF-β. Transient transfection assays in macrophages showed that Ets-2, similar to ESE-1 (13), increased NOS2 promoter activity. Induction of NOS2 by Ets-1 was modest, and NERF2 had no effect (Fig. 1). In contrast, Elk-3 markedly repressed NOS2 promoter activity (Figs. 1 and 2), and overexpression of Elk-3 blunted the induction of endogenous NOS2 by proinflammatory mediators (Fig. 3). Among the Ets family of proteins, Elk-3 is a member of the TCF subfamily, and it is known to have transcriptional repressive activity (18–22). Two important inhibi-
The presence of LPS 100 ng/ml was set as 100% (mean ± S.E.). This experiment was performed three times. For Elk-3, *, p compared with no LPS, and the signal intensities for NOS2 were plotted as indicated. Total RNA was extracted 6 h after vehicle or LPS administration, and Northern blot analyses were performed as described under “Experimental Procedures,” using 32P-labeled Elk-3, NOS2, and 18 S rRNA probes. B, the relative expression levels of Elk-3 (black bars) and NOS2 (white bars). The signal intensity of Elk-3 expression in the absence of LPS was set as 100%, and the signal intensity of NOS2 in the presence of LPS 100 ng/ml was set as 100% (mean ± S.E.). The signal intensities for Elk-3 were plotted as percentage of change when compared with no LPS, and the signal intensities for NOS2 were plotted as percentage of change when compared with 100 ng/ml LPS (mean ± S.E.). This experiment was performed three times. For Elk-3, * p < 0.05 versus no LPS treatment. For NOS2, † p < 0.05 versus no LPS treatment. This experiment was performed three independent times.

**Fig. 6.** Expression of Elk-3 inversely correlates with NOS2 mRNA in macrophages after LPS stimulation. As shown in A, RAW 264.7 cells were treated with vehicle or increasing doses of LPS as indicated. Total RNA was extracted 6 h after vehicle or LPS administration, and Northern blot analyses were performed as described under “Experimental Procedures,” using 32P-labeled Elk-3, NOS2, and 18 S rRNA probes. B, the relative expression levels of Elk-3 (black bars) and NOS2 (white bars). The signal intensity of Elk-3 expression in the absence of LPS was set as 100%, and the signal intensity of NOS2 in the presence of LPS 100 ng/ml was set as 100% (mean ± S.E.). The signal intensities for Elk-3 were plotted as percentage of change when compared with no LPS, and the signal intensities for NOS2 were plotted as percentage of change when compared with 100 ng/ml LPS (mean ± S.E.). This experiment was performed three times. For Elk-3, * p < 0.05 versus no LPS treatment. For NOS2, † p < 0.05 versus no LPS treatment. This experiment was performed three independent times.

**Fig. 7.** Expression of Elk-3 inversely correlates with NOS2 mRNA in tissues from mice with endotoxemia. Male C57BL/6 mice were injected intraperitoneally with LPS (10 mg/kg) or vehicle (saline). The mice were killed at 4 or 12 h after vehicle or LPS administration, and total RNA was extracted from kidney, lung, and heart tissues. Northern blot analysis was performed as described under “Experimental Procedures,” using 32P-labeled Elk-3, NOS2, and 18 S rRNA probes. This experiment was performed two independent times.

### Experimental Procedures

Male C57BL/6 mice were injected intraperitoneally with LPS (10 mg/kg) or vehicle (saline). The mice were killed at 4 or 12 h after vehicle or LPS administration, and total RNA was extracted from kidney, lung, and heart tissues. Northern blot analysis was performed as described under “Experimental Procedures,” using 32P-labeled Elk-3, NOS2, and 18 S rRNA probes. This experiment was performed two independent times.

### Adverse Effects of Elk-3 on LPS-Induced Gene Expression

TGF-β1 is a pleiotropic growth factor involved in a broad spectrum of cellular activities, including the modulation of immune function (27). Mice deficient in TGF-β1 exhibit widespread, uncontrolled inflammation and tissue necrosis, leading to death (28, 29). These data suggest that TGF-β1 is an important anti-inflammatory mediator. Furthermore, TGF-β1 is known to decrease macrophage responsiveness to LPS (30) and to prevent NOS2 expression in response to proinflammatory cytokines (14, 31–33). We have shown previously that TGF-β1 inhibited NOS2 induction at the level of gene transcription (14), and this effect occurred in part by down-regulating the expression of the architectural transcription factor high mobility group (HMG)-I/Y protein (17). In addition, we demonstrated that the administration of TGF-β1 to rats inhibited NOS2 expression in vivo and that TGF-β1 arrested LPS-induced hypotension and decreased mortality (16). Since our current data revealed that during an LPS response, endogenous expression of Elk-3 inversely correlated with NOS2 expression (Figs. 6 and 7), we wanted to determine whether the anti-inflammatory mediator TGF-β1 would alter the expression of Elk-3 during an inflammatory stimulus such as LPS. In the present study, TGF-β1 significantly increased Elk-3 mRNA levels that had been down-regulated by LPS in macrophages. This increase in Elk-3 correlated with a TGF-β1-induced down-regulation of NOS2 (Fig. 8). Our data suggest that TGF-β1 induction of Elk-3 is another mechanism by which TGF-β1 may down-regulate NOS2 gene transcription after its induction by proinflammatory stimuli.

The present study provides further insight into the regulation of the NOS2 gene by Ets factors. Ets-2 is able to increase NOS2 promoter activity; however, more impressively, Elk-3 is a potent repressor of NOS2 gene transactivation. Overexpression of Elk-3 can even suppress endogenous NOS2 that is induced by LPS and proinflammatory cytokines. The ability of Elk-3 to repress NOS2 depends on an inhibitory domain (NID, not CID) and the Ets domain of Elk-3. Moreover, endogenous Elk-3 mRNA is rapidly down-regulated by LPS, and this down-regulation precedes the induction of NOS2. Conversely, endogenous Elk-3 mRNA is induced by TGF-β1, and this induction is associated with a down-regulation of NOS2. Our data demonstrate that Elk-3 is a repressor of the NOS2 gene and that endogenous expression of Elk-3 inversely correlates with NOS2 expression. Thus, we propose that Elk-3 contributes to the tight control of NOS2 gene regulation in the setting of pro- and...
anti-inflammatory stimuli and that Elk-3 may serve as an important mediator of the NOS2 gene.

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