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Heme oxygenase-1 (HO-1) is a cytoprotective enzyme that is acutely induced by inflammatory stimuli, and the products of HO-1-mediated heme degradation have anti-inflammatory properties. In many different pathophysiologic states, the up-regulation of HO-1 has been shown to be beneficial in combating the detrimental consequences of increased inflammation. Ets transcription factors are known to be important mediators of inflammatory responses, and the ternary complex factor subfamily of Ets proteins has both transcriptional activation and repression activity. The present study demonstrates that of several ternary complex factor subfamily members, only Elk-3 represses HO-1 promoter activity in macrophages. Endotoxin administration to macrophages led to a dose-dependent decrease in endogenous Elk-3 mRNA levels, and this reduction in Elk-3 preceded the LPS-mediated up-regulation of HO-1 message. Analogous results also occurred in lung tissue of mice exposed to endotoxin. Two putative Ets binding sites (EBS1 and EBS2) are present in the downstream region of the murine HO-1 promoter (bp −125 and −93, respectively), and we recently showed that the EBS2 site is essential for HO-1 induction by endotoxin. In contrast, the present study demonstrates that the repressive effect of Elk-3 on HO-1 promoter activity is dependent on the EBS1 site. Taken together, our data reveal that Elk-3 serves as an important repressor of HO-1 gene transcription and contributes to the tight control of HO-1 gene regulation in the setting of inflammatory stimuli.

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The heme oxygenase (HO)4 enzyme system catalyzes the initial and rate-limiting step in heme degradation (1, 2). Three different isoforms of HO have been described, but HO-1 is the inducible isoform expressed in many cell types in response to pathophysiologic stimuli such as inflammation and endotoxin exposure (1, 3–10). During the process of heme degradation, equimolar concentrations of carbon monoxide (CO), biliverdin, and ferrous iron are produced (1, 2). Biliverdin reductase subsequently converts biliverdin into bilirubin. Interestingly, these downstream products of heme degradation have both anti-inflammatory and antioxidant properties (11–13). Thus, it appears that not only is HO-1 an acute indicator of an inflammatory response by its up-regulation, but the products of HO-1-mediated heme degradation (particularly CO and bilirubin) act in a compensatory manner to inhibit the inflammatory response or the consequences of increased oxidative stress. The importance of HO-1 during endotoxemia was underscored by our previous work showing increased mortality, end-organ damage, and oxidative stress in HO-1-null mice exposed to endotoxin (14, 15). These data together with previous work demonstrating an increase in HO-1 by endotoxin exposure suggest that induction of endogenous HO-1 counteracts the increased inflammation and oxidative injury associated with endotoxemia (6, 16–19).

Although great emphasis has been placed on the up-regulation of HO-1 and the beneficial effects of the products of heme degradation during disease processes or pathophysiologic stimuli involving oxidative stress, less is known about the repression of HO-1. We have previously shown in vascular smooth muscle cells that TGF-β1, a modulator of immune-mediated inflammatory responses, is able to suppress HO-1 induction by the proinflammatory cytokine IL-1β in vitro and LPS in vivo (17). Investigators have also found that the heme-binding factor, Bach1, is a repressor of HO-1 transcription (20). The target of HO-1 enzyme activity, heme, has been shown to abrogate the repression of Bach1 by inhibiting its binding to DNA in the enhancer regions of the HO-1 5′-flanking sequence (20). This inhibition of Bach1 binding allows activators of HO-1, such as Nrf2, to bind to these elements (21) and provide a feedback loop for the regulation of HO-1 expression. Another inducer of HO-1, cadmium, has been shown to activate the nuclear export of Bach1 and thus relieve Bach1-mediated repression of HO-1 gene regulation (22). Our present goal is to elucidate other potential regulatory pathways that may contribute to HO-1 repression and examine this regulation during an inflammatory stimulus.

The Ets family of proteins has been reported to be important in the modulation of mammalian immunity (23–25). Recently, we have demonstrated that two Ets family members associated with activation of gene transcription, principally Ets-2, but also Ets-1, contribute to the LPS induction of HO-1 by endotoxin exposure in macrophages (26). Previous investigations have also shown in Xenopus oocytes that Erg, Fli, and Ets-1 are capable of inducing

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4 Abbreviations used in this paper: HO, heme oxygenase; CO, carbon monoxide; EBS, Ets binding site; m, mutant; TCF, ternary complex factor subfamily; WT, wild type.

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HO-1 (27). Interestingly, Ets family members, including the ternary complex factors (TCF) subfamily, also have transcriptional repressive activity (28–32). Members of the TCF subfamily of Ets proteins include Elk-1, Elk-3, and Sap-1a. Elk-3, more than Elk-1, has been shown to be a repressor of gene transcription in the absence of MAPK activation (28, 33). Recently, we have demonstrated that Elk-3 repressed NO synthetase-2 expression during LPS exposure in macrophages (34). Thus, the goal of this study was to determine new pathways for HO-1 gene repression during an inflammatory stimulus, particularly involving the Ets family member Elk-3.

Materials and Methods

Cell culture and reagents

Murine macrophages (RAW 264.7) were grown according to the recommendations of American Type Culture Collection (Manassas, VA) as described previously (35). LPS from Escherichia coli (serotype O26:B6) was purchased from Sigma-Aldrich.

Plasmid constructs and cDNA probes

The luciferase reporter-promoter plasmids of HO-1 were generated by subcloning these fragments into the plg2L2-Vector (Promega) as previously described (18, 26). TCF expression plasmids were gifts from Dr. F. M. Stanley (New York University School of Medicine, New York, NY; pcDNA3-Elk-1 and pcDNA3-Sap-1a). ELK-3ΔA (Elk-3 without the A domain) in pCMV/Myc/nuc expression vector (Invitrogen Life Technologies) was generated as previously described (34). All constructs were verified by sequencing.

Site-directed mutagenesis

Mutants of Els binding sites (EBS) at bp −125 (mEBS1) and −93 (mEBS2) were generated by site-directed mutagenesis of the HO-1 (−295/ +74) plasmid using Pfu polymerase (Stratagene). In brief, PCR primers encoding mutant EBS, −122 to −125 and −90 to −93, were generated with CGTA substituted for ATCC in mEBS1 (5′-GAGTTATATGGTCTGGCATCCAGCGAG-3′) and 5′-CTGCTGTTGCAAGACAGAGATCATATGACTC-3′ and GTTT substituted for GGAT in mEBS2 (5′-CCCGGCTTTTGTTGGCAAGAC-3′). Underlining indicates mutated sequences. PCR was performed with Pfu polymerase using the wild-type HO-1 (−295/ +74) plasmid as a template. To generate mutant mEBS1/mEBS2, GGAT of EBS2 was replaced by ATCC of EBS1. Mutant construct mEBS1 was used as a template for PCR. The PCRs were digested with DpnI, and the undigested plasmids were transformed into XL2-Blue bacteria (Stratagene). Individual plasmids were sequenced to verify incorporation of the Ets site mutation.

Transient transfection and reporter activity assays

Transient transfection assays were performed using FuGene 6 transfection reagent (Roche). The HO-1 promoter-reporter plasmid (250 ng/well) plus the indicated amounts of Elk-1, Sap-1a, various Elk-3 expression constructs, and empty vector were cotransfected into murine macrophages. For assays in RAW 264.7 cells, 3 × 105 cells/well were plated in triplicate on six-well plates and incubated for 24 h. Twenty-four hours later, LPS (500 ng/ml) was given to the indicated wells. The cells, incubated with or without LPS for 24 h, were harvested for luciferase activity assays. Luciferase activity was measured by the Luciferase Assay System (Promega). Because many commonly used viral promoters contain potential binding sites for Ets factors, leading to potential artifacts using cotransfection of a second plasmid to determine transfection efficiency (36), we omitted this evaluation.

Mouse model of endotoxemia

Wide-type C57BL/6 mice were injected i.p. with LPS (10 mg/kg). Lungs were harvested at baseline and after 1, 2, and 4 h of LPS stimulation. All endotoxemia experiments in mice were performed in accordance with National Institutes of Health guidelines and were approved by Harvard Medical Area standing committee on animals.

RNA isolation and Northern blot analysis

Extraction of total RNA from cultured cells and mouse tissues was performed using the MINI RNA isolation kit (Qiagen). Total RNA (10 μg) was denatured, fractionated on 1.3% formaldehyde-agarose gels, and subsequently transferred to NitroPure filters (GE Osmonics). The filters were then hybridized with random-primed, [α-32P]dCTP-labeled HO-1 and Elk-3 cDNA probes. To correct for the differences in RNA loading, blots were subsequently hybridized to a 32P-labeled oligonucleotide probe complementary to 18S rRNA. The blots were exposed to a phosphoscreen and x-ray film. Radioactivity was measured using a PhosphorImager using ImageQuant software (Molecular Dynamics).

DNA-protein binding assay

Microinjection capture experiments were performed as described previously (26). In brief, RAW 264.7 cells were stimulated with vehicle or LPS (500 ng/ml) for 3 h, and nuclear extracts were extracted. The biotinylated EBS1, mEBS1, and EBS2 double-stranded oligonucleotides were used as probes. After adding 100 μg of streptavidin magnetic beads (New England Biolabs) and incubating at room temperature for 10 min with biotinylated probes, the tubes were clamped against a magnetic bar and washed twice with 500 μl of ice-cold binding buffer (20 mM HEPES, 50 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 0.5 mM KCl, 1 mM EDTA, 5 μM MgCl2, and 0.1 μM protease inhibitors). SDS-PAGE protein sample buffer was added, and after heating the samples to 95°C for 10 min, they were fractionated on a 4–12% gradient SDS-polyacrylamide minigel at 200 V for 1.5 h. Western blot analysis was then performed with a rabbit polyclonal Ab that we generated against aa 392–409 (SRLRSPVPLLSPSSQKS) of mouse Elk-3 (Zymed Laboratories).

Chromatin immunoprecipitation (ChIP) assay

The formaldehyde cross-linking and immunoprecipitation experiments were performed using the EZ ChIP chromatin immunoprecipitation kit (Upstate Cell Signaling Solutions). RAW 264.7 cells were treated with vehicle or LPS (500 ng/ml) for 3 h and fixed by addition of 1% formaldehyde to the medium for 10 min. Free formaldehyde was quenched by addition of 1× glyoxyl for 5 min at room temperature. The cells were washed twice with cold PBS, then cold PBS containing Protease Inhibitor Cocktail II was added, followed by harvesting of the cells by scraping and centrifugation. The cells were resuspended in 0.7 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris (pH 8.1)) supplemented with Protease Inhibitor Cocktail II (Roche). Then 0.4 ml of the cells were sonicated 25 times for 15 s each time, the lysates were cleared by centrifugation, and 5 μl of the sheared DNA was analyzed by agarose gel analysis. One hundred microliters of the sheared DNA was used for dilution buffer (0.1% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), and 167 mM NaCl). Chromatin solution was precleared for 1 h at 4°C with 60 μl of protein G-agarose/salmon sperm DNA. Ten microliters of the precleared chromatin solutions was saved for assessment of input chromatin, and the rest of the precleared chromatin solutions was incubated with 10 μl of preimmune serum or anti-Elk-3 Ab overnight at 4°C. Immune complexes were captured with 0.5 μl of protein G-agarose/salmon sperm DNA and 15 μl Protein G Plus/Protein A-Garose (Bio-chem) beads. Beads were washed sequentially: once in low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.1), and 150 mM NaCl), three times in high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.1), and 500 mM NaCl), twice in LiCl immune complex wash buffer (0.25 M LiCl, 1% Igepal-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, and 10 mM Tris (pH 8.1)), and twice in TE buffer (10 mM Tris-HCl and 1 mM EDTA (pH 8.0)). The Ab/agarose complexes were incubated twice with 100 μl of elution buffer (1% SDS and 100 mM NaHCO3) at room temperature for 15 min, and the eluates were combined. Cross-links of protein/DNA complexes were reversed by heating at 65°C overnight with 8 μl of 5 M NaCl. Reversed protein/DNA complexes were incubated with 5 μl of RNase A for 30 min at 37°C, then digested with 4 μl of 0.5 M EDTA, 8 μl of Tris-HCl, and 1 μl of protease K for 2 h at 45°C. The DNA was purified using a PCR purification kit (Qiagen) with 40 μl of distilled water. Three microliters of immunoprecipitated DNA was used for PCR analysis in 50-μl total reaction volumes, and the following primers were used in the ChIP assays: HO-1 (−317), 5′-CTG CCA AGA GCC AAC TCA TTA ATG TTT CTG C-3′, and HO-1 (−25), 5′-CTT TAA GCA CCG GGG TCA CGT GGT G-3′. PCR conditions were as follows: 30 cycles of 94°C for 3 min, 58°C for 45 s, and 72°C for 45 s. Five microliters of PCR products were analyzed by 7% PAGE gel, resulting in a 292-bp fragment.
Statistics

Where indicated, comparisons between groups were made by factorial ANOVA, followed by Fisher’s least significant difference test. Statistical significance is accepted at \( p < 0.05 \).

Results

Elk-3 is down-regulated by LPS in lung tissue in vivo and in macrophages in vitro

The lung is an organ often injured during a systemic inflammatory response, and HO-1 is induced in lung tissue after exposure to endotoxin (14, 17). Thus, to determine the regulation of Elk-3 mRNA in vivo and to assess its temporal expression pattern compared with HO-1, we administered endotoxin (LPS) to mice and then harvested lung tissues at different time points for total RNA extraction. Northern blot analysis showed that the expression of Elk-3 was decreased in lung tissue as early as 1 h after LPS stimulation and continued to decrease during the first 4 h of LPS exposure (Fig. 1). At the point in time that Elk-3 began to be suppressed by LPS in lung tissue, HO-1 mRNA began to increase (1 h).

The lung is an organ rich in macrophages, even under basal conditions, and this inflammatory cell contributes to the pathophysiology of endotoxin-induced injury. We next harvested total RNA from mouse macrophages (RAW 264.7 cells) that were treated with increasing doses of LPS (0, 1, 10, and 100 ng/ml) for 1, 4, and 8 h. By Northern blot analysis, we found that mRNA levels of Elk-3 decreased by 4 h at even the lowest dose of LPS (1 mg/ml), and this repression of Elk-3 message was more complete with doses of 10 and 100 ng/ml (Fig. 2). This LPS effect occurred by 4 h, a point in time that precedes any significant HO-1 mRNA induction. By 8 h after LPS stimulation, Elk-3 message remained reduced (most completely with the dose of 100 ng/ml), whereas HO-1 mRNA was being induced by LPS (dose dependently) in macrophages. These data together with the lung expression studies demonstrate that endogenous expression of Elk-3 inversely correlates with HO-1 expression after exposure to LPS (in vivo and in vitro).

Elk-3 represses HO-1 promoter activity

To understand the role of Ets subfamily members, especially TCFs, due to their potential as both positive and negative mediators of gene transcription (28–32), we investigated the effects of Elk-3, Elk-1, and Sap-1a on the regulation of HO-1 promoter activity. RAW 264.7 cells were transfected with expression plasmids for Elk-3, Elk-1, and Sap-1a, and HO-1 promoter activity (construct −4045 to +74) was assessed in the presence or the absence of LPS (Fig. 3A). We found that Elk-1 and Sap-1a increased HO-1 promoter activity, but much less than stimulation with LPS. However, Elk-3 showed a strong repressive effect (>80% repression) on the activity of the HO-1 promoter under basal conditions (Fig. 3A). Moreover, this repressive effect of Elk-3 overexpression on HO-1 promoter activity was evident even during LPS stimulation in macrophages (Fig. 3A). By increasing the amount of expression plasmid, Elk-3 was able to completely reverse the induction of HO-1 promoter activity by LPS in a dose-dependent manner (Fig. 3B). These data are consistent with those in Figs. 1 and 2 showing that the expression of Elk-3 is inversely related to the expression of HO-1. LPS stimulation combined with overexpression of Elk-3 produced additional transactivation of the HO-1 promoter, whereas overexpression of Sap-1a had no additional effect on LPS-stimulated HO-1 promoter activity in macrophages (Fig. 3A). These data suggest that Elk-3 is unique among the TCF subfamily members as a strong repressor of HO-1 promoter activity even during an inflammatory stimulus in macrophages.

Elk-3 DNA binding domain is critical for repression of HO-1 transactivation

To determine whether the Elk-3 repressive effect was dependent upon its binding to the HO-1 promoter, we repeated the transient transfection experiments using an Elk-3 expression construct devoid of the Ets (A) domain, which is responsible for Elk-3 binding to DNA. Fig. 4 demonstrates that the ability of Elk-3 to repress HO-1 transactivation under basal conditions or during LPS stimulation is abolished in the absence of the Ets (A) domain. These data suggest that the DNA binding domain of Elk-3 is necessary to produce its suppressive effect on the HO-1 promoter.

Elk-3 binding in the downstream HO-1 promoter (EBS1) is critical for its repressive effect

We recently demonstrated that of the Ets binding sites in the downstream region of the HO-1 promoter (EBS1 and EBS2), the EBS2 site is critical for activation of the HO-1 promoter during endotoxin exposure in macrophages (26). Thus, to determine whether these Ets binding sites are involved in Elk-3 repression, we performed transient transfection experiments using either a wild-type (WT) HO-1 promoter construct or constructs containing mutations in EBS1 (mEBS1) or EBS2 (mEBS2). We have recently shown that promoter activity within construct −295/+74 maintains activity analogous to construct −4045/+74. Thus, EBS1 and EBS2 were mutated within construct −295/+74. These constructs were cotransfected with increasing concentrations of Elk-3 expression plasmid (50 or 200 ng/well). Mutation of EBS1 (mEBS1) abrogated the suppressive effect of Elk-3 on HO-1 promoter activity (both 50 and 200 ng), whereas repression of HO-1 promoter activity was dramatic in the mEBS2 construct (Fig. 5A). These data suggest that in contrast to activators of HO-1 promoter activity that...
The binding of Elk-3 is decreased by 67%. Moreover, when EBS1 site under basal conditions; however, in the presence of LPS, the binding of Elk-3 is decreased by 88% or the absence (71%) of LPS. Elk-3 is also capable of binding at the EBS2 site, but the efficiency is <50% of the binding at the EBS1 site.

To verify that the repressive activity of Elk-3 occurs by its direct binding to the EBS1 site, we performed a DNA-protein binding assay using a microaffinity capture technique (26). EBS1, mutant EBS1 (mEBS1), and EBS2 probes were incubated with nuclear extracts from RAW 264.7 cells treated with vehicle or LPS for 3 h. The bound proteins were recovered, and Elk-3 was assessed by Western blot analysis. Fig. 5A demonstrates that we were starting with comparable amounts of chromatin, input lanes are shown to display the equivalent concentration of DNA used for this experiment (Fig. 5A). When preimmune serum was used (as a negative control) instead of the Elk-3 Ab, no binding was apparent (lane 3). To demonstrate that we were starting with comparable amounts of chromatin, input lanes are shown to display the equivalent concentrations of DNA used for this experiment (Fig. 5C).

Finally, to confirm the importance of the EBS1 site in the repression of HO-1 by Elk-3, we generated an additional construct with the EBS2 site replaced by the EBS1 site. Fig. 6A shows a schematic diagram of the constructs that include 1) WT (intact EBS1 and EBS2 sites), 2) mEBS1 (mutation of the EBS1 site and a WT EBS2 site), and 3) mEBS1/EBS1 (mutant EBS1 site and EBS2 replaced by EBS1). The mutant and WT HO-1 promoter constructs were cotransfected with the expression plasmid for Elk-3 or an equivalent amount of empty vector DNA. Confirming the data in Fig. 5, Elk-3 repressed WT HO-1 promoter activity, and mutation of the EBS1 site (mEBS1) prevented this repression. When the EBS2 site was replaced by EBS1 in the mEBS1 construct (mEBS1/EBS1), the repressive effect of Elk-3 was restored (Fig. 6B). These data confirm that EBS1 is essential for the repression activity of Elk-3.

EBS1 modulates HO-1 transactivation by LPS

To elucidate the importance of the EBS sites on HO-1 transactivation in the presence or the absence of LPS, we performed additional cotransfection experiments using the WT and mutant HO-1 promoter constructs depicted in Fig. 6A. Mutation of the EBS1 site (mEBS1), which is responsible for Elk-3 repression, led to a significant reduction in HO-1 promoter activity. In contrast, mutation of both EBS1 and EBS2 sites (mEBS1/EBS2) resulted in a complete loss of HO-1 promoter activity, indicating the essential role of the EBS1 site in Elk-3 repression.

FIGURE 3. TCF transcription factors regulate HO-1 promoter activity in macrophages. A, Luciferase reporter plasmid HO-1(–4045/+74) (250 ng/well) and TCF expression plasmids for Elk-3, Elk-1, and Sap-1a (200 ng/well) were transiently cotransfected into RAW 264.7 cells. Empty pCI vector was added as a control and to keep the total plasmid DNA content constant. After transfection, cells were treated with vehicle or LPS (500 ng/ml) for 24 h, then harvested. Luciferase activity was plotted as fold induction of HO-1 promoter activity from cells receiving no TCF expression plasmids and no LPS. This experiment was performed twice (n = 6 in each experiment). *, p < 0.05 vs control, Elk-1, and Sap-1a in each group. B, The HO-1 luciferase reporter plasmid was cotransfected in RAW 264.7 cells along with increasing concentrations of expression plasmid for Elk-3, as shown. After transfection, cells were treated with vehicle or LPS (500 ng/ml) for 24 h, then harvested. Luciferase activity was plotted as fold induction of HO-1 promoter activity from cells receiving no Elk-3 expression plasmid and no LPS. This experiment was performed twice (n = 6 in each experiment).

FIGURE 4. Role of EtsA domain in HO-1 repression by Elk-3. RAW 264.7 cells were transiently cotransfected with luciferase reporter plasmid HO-1(–4045/+74) (250 ng/well) along with wild-type Elk-3, Elk-3 with a deletion mutant of the A domain (Elk-3ΔA), or pCI control plasmid (all at a concentration of 200 ng/well). Luciferase activity was presented as fold induction of HO-1 promoter activity compared with cotransfection with control plasmid. *, p < 0.05 vs control and Elk-3ΔA. This experiment was performed twice (n = 6 in each experiment).
to a significant increase in HO-1 promoter activity at baseline compared with the WT construct (Fig. 7A). When the EBS2 site was replaced by EBS1 in the mEBS1 construct (mEBS1/EBS1), baseline promoter activity returned to the level of the WT construct. Next, we assessed HO-1 promoter activity in the presence of LPS. Using the WT construct, promoter activity increased 5-fold in the presence of LPS (Fig. 7A). Mutation of the EBS1 site (mEBS1) in the presence of an intact EBS2 site led to enhanced induction of HO-1 promoter activity by LPS. Replacement of EBS2 by EBS1 in the mEBS1 construct (mEBS1/EBS1) diminished the enhanced transactivation by LPS (Fig. 7A). These data are consistent with our previous findings that the EBS2 site, more than the EBS1 site, is essential for the most potent transactivation of the HO-1 promoter. In addition, these data suggest that a suppressive effect through the EBS1 site occurs under basal conditions, and mutation of this EBS1 site allows potentiated induction of the HO-1 promoter by LPS. Combining the results of the present study with our recent findings on transactivation of HO-1 by Ets-2 (26), we propose a schema depicting the transcriptional regulation of HO-1 by the Ets proteins Elk-3 and Ets-2 (Fig. 7B). This schema will be described in detail in the Discussion.

Discussion

In many different pathophysiologic circumstances, the up-regulation of HO-1 has been shown to be beneficial in combating the detrimental consequences of increased inflammation and oxidative stress (6, 10, 37–41). However, recent evidence suggests that increased levels of HO-1 can also have detrimental consequences in certain disease processes, such as accelerating tumor angiogenesis (42). Consistent with this observation, inhibitors of HO have been shown to have antitumor activity (43, 44). These data suggest that under specific circumstances, inhibition of HO can also have beneficial effects. Thus, additional investigations are necessary to understand the regulation of HO-1, not only activation but also suppression, and to determine the physiologic consequences of these modifications.

The TCF subfamily of Ets factors is known to have both transcriptional activation and repression activity depending on the biological setting. For instance, in the absence of MAPK signaling, predominantly Elk-3 (and, to a lesser extent, Elk-1) is capable of repressing gene transcription (33). Interestingly, Elk-3 does not always repress gene transcription, because activation of ERK can convert Elk-3 into a transcriptional activator by Ras. Because of the established link between MAPK signaling and inflammation (45), including the initiation phase of innate immunity, we wanted to determine the role of TCF subfamily members, particularly
Elk-3, in HO-1 gene regulation during an inflammatory stimulus in macrophages.

We have previously noted that Elk-3 is down-regulated during an inflammatory stimulus in cultured macrophages (34). Thus, to investigate the temporal relationship between Elk-3 and HO-1 promoter regulation during endotoxin exposure, we assessed mRNA levels for both Elk-3 and HO-1 in mouse tissue and cells. After LPS exposure, an inverse relationship between Elk-3 and HO-1 was noted in vivo (lung tissue; Fig. 1) and in vitro (macrophages; Fig. 2). To evaluate the functional significance of this inverse relationship, Elk-3 was overexpressed in mouse macrophages, and HO-1 promoter activity was analyzed in the presence or the absence of LPS stimulation. Elk-3 represses the promoter activity of HO-1 through the EBS1 site (left). After LPS stimulation, the expression level of Elk-3 and its ability to bind to EBS1 decrease, whereas Ets-2 expression and its binding to the EBS2 site increase, resulting in induction of HO-1 and enhanced gene expression (right).

Elk-3 binds with high affinity at EBS1 (Fig. 5B), whereas binding at EBS2 is weaker. Furthermore, LPS stimulation led to decrease in Elk-3 binding (Fig. 5B). Interestingly, our recent study showed that in contrast to Elk-3, an activator of HO-1 transcription, Ets-2, binds with high affinity at the EBS2 site after LPS stimulation (26). The significance of the Elk-3 binding was confirmed in vivo, because ChIP assays revealed that Elk-3 binds in this downstream region of the HO-1 promoter at baseline, and LPS stimulation led to a decrease in Elk-3 binding (Fig. 5C). Taken together, these data suggest that of the two Ets binding sites in the downstream HO-1 promoter, EBS1 functions predominantly to bind the repressor Elk-3 under basal conditions.

To confirm the functional roles of the two Ets binding sites, we performed mutation studies of the HO-1 promoter. Fig. 5A reveals that mutation of the EBS1 site prevented repression of the HO-1 promoter by Elk-3, whereas mutation of the EBS2 site allowed a persistent and dramatic down-regulation of the HO-1 promoter by Elk-3. In the construct containing an mEBS1 site, we replaced the EBS2 site with the EBS1 site. This switch in transcription factor binding sites rescued the repressive action of Elk-3 (Fig. 6). These data confirm that EBS1, not EBS2, is responsible for the repressive action of Elk-3. Bach1 is another DNA-binding molecule that acts to repress HO-1 transcription (20, 21). Previous studies have shown that Bach1 inhibits HO-1 transcription by binding stress response elements in the two upstream enhancer regions (−10 and −4 kb) of the HO-1 5′-flanking sequence (20). Thus, our present study provides the first evidence of a repressive transcription factor, Elk-3, that functions through binding at a downstream site in the HO-1 promoter (EBS1; at bp −93).

Finally, we wanted to characterize these downstream Ets binding sites in the presence or the absence of LPS stimulation. Mutation of the EBS1 site, responsible for Elk-3 repression led to a significant increase in promoter activity at baseline compared with the WT construct (Fig. 7A). These data confirm that EBS1 is responsible for a baseline repression of HO-1 promoter activity. Mutation of EBS1 in the presence of an intact EBS2 led to enhanced activation of the HO-1 promoter by LPS (Fig. 7A). Replacement of this EBS2 site with EBS1 diminished the enhanced transcriptional activity of HO-1 promoter by LPS. These data are consistent with our previous finding that EBS1 is not as important as EBS2 for transcriptional regulation of the HO-1 promoter by LPS (26). In addition, these data suggest that a basal suppressive effect through the EBS1 site is able to dampen LPS-induced transcriptional activity of the HO-1 promoter.

The present study provides additional insight into regulation of HO-1 gene expression, both at baseline and during an inflammatory stimulus. Taking into account the present data and our recent studies assessing the role of Ets factors that induce HO-1 gene expression (particularly Ets-2) (26), we propose the schema in Fig. 7B. This schema depicts the transcriptional regulation of HO-1 by Ets proteins. Elk-3 binding to the EBS1 site keeps the transcriptional activity of HO-1 at a lower level under basal conditions (left). When cells are exposed to the inflammatory stimulus, LPS, Elk-3 expression and binding to the EBS1 site decrease, thus releasing its basal suppressive effect. At this same time during LPS exposure, the expression level of Ets-2 and its binding to the EBS2 site increase (right). This schema demonstrates how positive and negative mediators of HO-1 gene expression are able to regulate the transcriptional activity of the HO-1 promoter during an inflammatory stimulus in macrophages. We propose that Elk-3 contributes to the tight control of HO-1 gene regulation in the setting of proinflammatory stimuli, and that Elk-3 may serve as an important mediator of HO-1 gene regulation.
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Disclosures

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References


