Role of Ets-2 in the Regulation of Heme Oxygenase-1 by Endotoxin*

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Ets proteins play a vital role in the regulation of mammalian immunity, and family members Ets-1 and Ets-2 regulate a variety of genes that participate in the propagation of an inflammatory response. Heme oxygenase (HO)-1, although acutely induced by inflammatory stimuli, has cytoprotective properties and prevents an exaggerated inflammatory response. Ets-1 and Ets-2 both induce HO-1 promoter activity; however, Ets-2 was a more potent transactivator of HO-1 in macrophages. A potent inflammatory mediator, bacterial lipopolysaccharide (LPS), induced Ets-2 at the mRNA and protein level, and this induction preceded the up-regulation of HO-1. To further delineate the role of Ets-2 in regulating HO-1 transcription, we performed HO-1 promoter analysis studies in macrophages. Deletion mutants down to −137/+74 maintained an activity analogous to that of the largest construct, −4045/+74. Further deletion constructs (starting with −117/+74) showed a significant reduction in promoter activity when co-transfected with Ets-2 or exposed to LPS. Promoter sequence analysis revealed two putative Ets binding sites (EBSs) in this region, and mutation of these sites showed that EBS −93, more than EBS −125, was critical for full HO-1 promoter activity. Additional studies showed that EBS −93 binds Ets-2 and that mutation of the DNA binding domain of Ets-2 entirely prevented transactivation of HO-1. Finally, overexpression of a dominant negative form of Ets-2 blunted HO-1 promoter induction by LPS, and kinase inhibitors (PI3K more than JNK) that reduced Ets-2 expression markedly decreased endogenous HO-1 expression. Our data provide evidence that Ets-2 contributes to the up-regulation of HO-1 by the potent inflammatory stimulus LPS in macrophages.

Heme oxygenase (HO) is the rate-limiting enzyme in heme breakdown to generate carbon monoxide (CO), biliverdin (subsequently reduced to bilirubin by biliverdin reductase), and free iron (sequestered by ferritin). Three different HO isoforms have been described, and HO-1 is the highly inducible isoform proposed to interact with transcription factors activated by inflammatory stimuli such as inflammation and endotoxin exposure (1–9). The importance of HO-1 during endotoxemia was demonstrated by the increased mortality, increased oxidative stress, and exaggerated end-organ damage in HO-1-null mice compared with wild-type mice (10, 11). These data, together with previous work demonstrating the induction of HO-1 by endotoxin exposure (12–14), suggest that induction of endogenous HO-1 occurs in an effort to counteract the increased inflammation and oxidative injury associated with endotoxia.

HO-1 and the subsequent metabolites of heme catabolism play vital roles in regulating inflammation, oxidative stress, and cell survival (15) in many animal models of disease. The proposed mechanisms by which HO-1 exerts its biological effects include the following: (a) the ability to degrade the potentially toxic heme; (b) the release of biliverdin and subsequent conversion to bilirubin, both of which have antioxidant properties (16); (c) the generation of CO, which has vasodilatory, antiproliferative, and anti-inflammatory properties (15); and (d) the induction of ferritin, which has iron-binding properties (17). Due to these beneficial effects of HO-1 during an inflammatory stimulus, we wanted to further understand the regulation of HO-1 gene expression during endotoxin exposure.

The Ets proteins are a family of transcription factors that are known to be involved with inflammatory responses (18–20) or to interact with transcription factors activated by inflammatory responses (21, 22). The Ets superfamily of transcription factors is composed of proteins that share a DNA binding motif (winged helix-turn-helix motif) that allows Ets proteins to interact with GGAA/T-containing DNA elements (18, 23). The majority of Ets family members bind to DNA as monomeric proteins; however, Ets-1 and Ets-2 have been shown to interact with a variety of transcription factors, including AP-1 and nuclear factor-κB, to regulate gene expression (22). In these circumstances, Ets factors often regulate the transcriptional activity of enhancer elements containing adjacent cis-acting elements, for example, Ets and AP-1 binding sites (21). Due to the presence of putative Ets binding domains in the −4 kb enhancer region of the mouse HO-1 gene, which are located in close proximity to stress response elements that contain functionally important AP-1 binding sites (14, 24, 25), we hypothesized that Ets family members may be important transcription factors driving HO-1 expression during an inflammatory response.

Deramaudt et al. (26) previously investigated the role of Ets factors, particularly Erg, in regulating HO-1 in oocytes and endothelial cells to promote angiogenesis. However, the importance of Ets factors in regulating HO-1 induction during an inflammatory stimulus (such as endotoxin exposure) is not known. Thus, due to the potential importance of Ets factors in immune cell signaling and vascular inflammation, we wanted to determine whether members of the Ets superfamily could regulate HO-1 gene expression.
EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Murine macrophages (RAW 264.7) were cultured as described previously (27). Lipopolysaccharide (LPS) from Salmonella typhosa was purchased from Sigma. Kinase inhibitors used in our studies were purchased from BIOMOL International L.P. (Plymouth Meeting, PA; SV240402, SP890519, and PD98059) were all used in concentrations of 5–10 μM.

Luciferase Reporter Constructs and Expression Plasmids—An HO-1 luciferase reporter plasmid, HO-1-(−4045/+74), was amplified from mouse genomic DNA by PCR and subcloned into the pGL2 luciferase reporter vector (Promega, Madison, WI). This HO-1 reporter was a gift from Dr. Shaw-Fang Yet. HO-1-(−3527/+74), HO-1-(−2986/+74), HO-1-(−1995/+74), HO-1-(−1377/+74), HO-1-(−117/+74), HO-1-(−66/+74), and HO-1-(−35/+74) deletion mutants were generated by PCR amplification with HO-1-(−4045/+74) as the template. Expression plasmids pcI-Ets-1, pcI-Ets-2, pcI-NERF2, and pcI-Elk-1 were a generous gift from Dr. Peter Oettgen. The dominant negative mutant of Ets-2 (28, 29) and Ets-2ΔDBD (30) were generated by PCR amplification from mouse Ets-2 cDNA. Ets-2 phosphorylation mutant with a substitution of alanine for the wild-type threonine at position 72, pcI-Ets-2T72A, was generated as described previously (31).

Site-directed Mutagenesis—Mutants of Ets binding sites (EBSs) at bp −125 (mEBS1), −93 (mEBS2), and double mutant (mEBS1+2) were generated by site-directed mutagenesis of the HO-1-(−4045/+74) plasmid using Pfu polymerase (Stratagene, La Jolla, CA). In brief, PCR primers encoding mutant EBSs, −122 to −125 and −90 to −93, were designed to substitute for ATCC in mEBS1 (5′-GAGCTCATATGATTTCTGACCAGCAG-3′ and 5′-CTGCGCTGTAAGTCTGAAAATCATATGACTC-3′) and GTTT substituted for GGAT in mEBS2 (5′-CTTCCGGCGTTGGTTGCAACAGCAG-3′ and 5′-CTGCTGTGACCAAAACCGCCGAGG-3′). PCR was performed with Pfu polymerase using the wild-type HO-1-(−4045/+74) plasmid as a template. The PCR was digested with DpnII, and the undigested plasmids were transformed into XL2-Blue bacteria (Stratagene). Individual plasmids were sequenced to verify incorporation of the Ets site mutation (schemeatic structures as described in the top panel of Fig. 4A).

Transient Transfections of RAW 264.7 Cells—HO-1 reporter constructs were used for transient transfection assays of RAW 264.7 cells as described previously (14), and 3 × 10⁵ cells/well were plated in triplicate on 6-well plates and incubated overnight. Using FuGENE 6 transfection reagent (Roche Applied Science), cells were transfected in triplicate on 6-well plates and incubated overnight. Using FuGENE 6 transfection reagent (Roche Applied Science), cells were transfected with 250 ng of a HO-1 reporter plasmid plus the indicated amounts of expression constructs, Ets-1, Ets-2 (wild-type or mutated forms), NERF2, and Elk-1. At least three independent experiments, carried out in triplicate, were performed for each experimental condition.

Electrophoretic Mobility Shift Assays and Microprotein Capture Experiments—Electrophoretic mobility shift assay was performed as described (32), using nuclear extracts from RAW 264.7 cells stimulated with vehicle or LPS (500 ng/ml) for 6 h. In brief, double-stranded oligonucleotide probes encoding region −99 to −82 (EBS2) of the HO-1 5′-flanking sequence (5′-CGGGCGGGCTGATTGTTGCAAC-3′; the Ets binding site is underlined) were used. The probe containing EBS at bp −93 was end-labeled with T4 polyadenylate kinase. Labeled DNA (approximately 50,000 cpm) was used in each binding reaction. Microprotein capture experiments were performed as described previously (33), with modifications. Purification of DNA-binding proteins by this microprotein technique was performed using the EBS2 double-stranded oligonucleotide probe, with the upper strand synthesized with a 5′-biotin modification. The probe was incubated with 200 μg of nuclear extract protein, under the same binding conditions used for the electrophoretic mobility shift assay, in volumes of 100–200 μl. After the addition of 100 μg of streptavidin magnetic beads (New England BioLabs) and incubation at 4 °C for 30 min, the tubes were clamped against a magnetic bar and washed twice with 500 μl of ice-cold nuclear extraction buffer. SDS-PAGE protein sample buffer was added, and after heating the samples to 95 °C for 10 min, they were fractionated on 12% SDS-polyacrylamide minigels. Western blot analysis was then performed with rabbit polyclonal anti-Ets-2. In experiments using recombinant Ets-2 protein, pcI-Ets-2 was used as a template for generating 35S-labeled in vitro translation products in the TnT quick coupled transcription/translation systems (Promega).

RNA Isolation and Northern Blot Analysis—RNAeasy mini RNA isolation kit (Qiaogen, Valencia, CA) was used to extract total RNA from cultured cells. Northern blot analysis was performed as described previously (10, 32). In brief, RNA samples (10 μg) were electrophoresed in a 1.5% agarose gel containing 3.7% formaldehyde and then transferred to NitroPure filters (GE Osmonics Inc., Minnetonka, MN). The filters were then hybridized with random primed, [α-32P]dCTP-labeled Ets-2 or HO-1 probes. To correct for the differences in RNA loading, blots were subsequently hybridized to a γ-32P-labeled oligonucleotide probe complementary to 28S rRNA. The blots were exposed to a phospho-screen and x-ray film. The radioactivity was measured on a PhosphorImager by using ImageQuant software (Amerham Biosciences).

Total Protein Isolation and Western Blot Analysis—Whole cell lysates (30 μg) of RAW 264.7 cells were boiled and resolved on 12% SDS-PAGE gel. Proteins were transferred on Pure nitrocellulose membranes and immunoblotted with SuperSignal® West Pico Chemiluminescent Substrate (Pierce). Rabbit polyclonal anti-Ets-2 (IC-351; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-HO-1 (Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada) antibodies were diluted to 1:2000 before use.

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

RESULTS

Ets-2 Activates HO-1 Promoter Activity—Due to the importance of the Ets family of proteins in immunologic responses (18–20), we postulated that Ets factors may also play a role in the regulation of HO-1 in response to an inflammatory stimulus. We initially examined the ability of a panel of macrophage-expressed Ets factors to transactivate the −4 kb HO-1 promoter. Mouse macrophages (RAW 264.7 cells) were transiently transfected with expression plasmids for Ets-1, Ets-2, NERF2, and Elk-1, and promoter activity of HO-1-(−4045/+74) reporter construct was assessed. We found that Ets-1 and Ets-2 increased HO-1 promoter activity (by 3-fold and by 7-fold, respectively; p < 0.05), whereas NERF2 and Elk-1 had no effect (Fig. 1). In addition, we found that LPS induced HO-1 promoter activity by 8-fold. To confirm the induction of HO-1 promoter activity by Ets factors, we co-transfected HO-1-(−4045/+74) reporter construct with increasing amounts of the Ets-2 expression plasmid and demonstrated that Ets-2 induces HO-1 promoter activity in a dose-dependent manner (data not shown). These results suggest that Ets-2 is a potential transcriptional regulator of the HO-1 promoter and that the ability of Ets-2 to transactivate HO-1 is comparable with LPS stimulation.

Induction of Ets-2 Precedes Induction of HO-1 by LPS Stimulation in Macrophages—To investigate the temporal relationship between Ets-2 and HO-1 expression, we treated RAW 264.7 cells with LPS (500 ng/ml) and harvested total RNA at 0.5, 1, 2, 6, 12, and 24 h after LPS. HO-1 mRNA levels began to
were performed as described under “Experimental Procedures.” After LPS treatment as indicated. Northern blot and Western analyses were extracted from RAW 264.7 cells at various time points. B, total RNA; 1 pCI-Ets-2 cells were treated with LPS (500 ng/ml). Total RNA (pCI; 1/H9262) was extracted from RAW 264.7 cells were transiently transfected with either an expression plasmid Ets-2-specific antibodies. C, RAW 264.7 cells were transiently transfected with either an expression plasmid encoding Ets-2 (pCI-Ets-2; 1 μg/well) or the corresponding empty vector (pCI, 1 μg/well). Cells receiving the same transfection reagents were also treated with vehicle (−, negative control) or LPS (10 ng/ml, positive control). Total protein was extracted 24 h after vehicle or LPS administration, and Western blot analysis was performed as described under “Experimental Procedures,” using the Ets-2-specific antibody.

increase by 6 h, and marked induction of HO-1 was evident after 12 h of LPS treatment (Fig. 2A). Ets-2 message was evident at baseline (vehicle-treated cells); however, induction of Ets-2 began by 1 h after LPS administration. To evaluate protein expression, we treated RAW 264.7 cells with LPS and harvested total protein at analogous time points. Ets-2 protein was seen as a doublet, with the upper band of the doublet representing the phosphorylated form of Ets-2 (Fig. 2B, P-Ets-2), as confirmed by the disappearance of this band after treatment of the extracted protein with alkaline phosphatase (data not shown). HO-1 protein levels were markedly increased 12 h after LPS treatment (Fig. 2B). Ets-2 protein levels began to increase 1 h after LPS treatment and were maximally induced after 6 h.

Also, to determine whether overexpression of Ets-2 could increase endogenous HO-1 expression, we transiently transfected RAW 264.7 cells with expression plasmids for Ets-2 (pCI-Ets-2) or empty plasmid (pCI). Cells receiving the same transfection reagents were also treated with vehicle (negative control) or LPS (positive control) for 24 h. Total protein was harvested from the cells, and Western blots were performed. Although only a portion of the cells were transfected with the Ets-2 expression plasmid (in contrast to the ubiquitous expression in LPS-stimulated cells), extraction of total protein revealed an increase in exogenous Ets-2 (Fig. 2C). Cells transfected with pCI-Ets-2 revealed an induction in endogenous HO-1, compared with no HO-1 induction in cells transfected with pCI (empty plasmid; Fig. 2C). Taken together, these data demonstrate that Ets-2 induction precedes HO-1 induction by LPS at both the mRNA and protein levels and that overexpression of Ets-2 alone is capable of inducing endogenous HO-1 expression.

**FIG. 2.** LPS induction of Ets-2 precedes HO-1 induction. RAW 264.7 cells were treated with LPS (500 ng/ml). Total RNA (A) and total protein (B) were extracted from RAW 264.7 cells at various time points after LPS treatment as indicated. Northern blot and Western analyses were performed as described under “Experimental Procedures.” A, 32P-labeled HO-1, Ets-2, and 28 S rRNA probes were used for Northern blot experiments. B, Western blot analyses were performed using HO-1- and Ets-2-specific antibodies. P-Ets-2 represents the phosphorylated form of Ets-2. Two independent experiments were performed. C, RAW 264.7 cells were transiently transfected with either an expression plasmid encoding Ets-2 (pCI-Ets-2; 1 μg/well) or the corresponding empty vector (pCI, 1 μg/well). Cells receiving the same transfection reagents were also treated with vehicle (−, negative control) or LPS (10 ng/ml, positive control). Total protein was extracted 24 h after vehicle or LPS administration, and Western blot analysis was performed as described under “Experimental Procedures,” using the Ets-2-specific antibody.

**FIG. 3.** HO-1 promoter has Ets-2 response sites downstream of bp −137. RAW 264.7 cells were transiently co-transfected with deletion mutants of the HO-1 promoter (250 ng/well) and pCI-Ets-2 expression vector (A) or empty pCI vector (B). After the transfection, cells were treated with vehicle (A) or LPS (500 ng/ml; B) for 24 h and then harvested. For A and B, HO-1 promoter activity was calculated by comparison with the HO-1(−4045/+74) promoter construct. *, p < 0.05 versus activity of HO-1(−4045/+74) promoter construct stimulated with either Ets-2 co-transfection or LPS treatment.

Ets-2- and LPS-responsive Elements Are Downstream of bp −137 in the HO-1 Promoter—Initially, we assessed the responsiveness of construct HO-1(−4045/+74) to stimulation by Ets-2 and LPS. This construct, containing the HO-1 −4 kb enhancer region, was induced a similar magnitude by Ets-2 and LPS, as shown in Fig. 1. To further localize the Ets-2- and LPS-responsive element(s), we made deletion mutants of HO-1(−4045/+74) and either co-transfected these deletion constructs with an expression plasmid for Ets-2 (Fig. 3A) or stimulated the constructs with LPS (500 ng/ml; Fig. 3B). The data are presented as fold induction of promoter activity compared with HO-1(−4045/+74) promoter construct in the absence of Ets-2 or LPS. Induction of the deletion mutants by Ets-2 or LPS was similar to construct HO-1(−4045/+74) down to construct HO-1(−137/+74). Further deletions (including constructs HO-1(−117/+74), HO-1(−66/+74), and HO-1(−34/+74)) showed a significant reduction in promoter activity when exposed to Ets-2 (Fig. 3A) or LPS (Fig. 3B). These results suggest that the cis-acting elements critical for HO-1 induction by either Ets-2 or LPS in these mouse macrophages are located downstream of bp −137 in the HO-1 promoter.

Ets Binding Site at bp −93 Is Critical for Induction of HO-1 Promoter Activity by Ets-2 and LPS—To identify the exact Ets-2- and LPS-responsive sites, we further analyzed the HO-1 promoter sequence downstream of bp −137. Sequence analysis of this region revealed putative Ets-2 binding sites at −125 and −93. We named these two sites EBS1 (5′-GATTATC-CCCTTA-3′) and EBS2 (5′-GGGCTGGATGTTGC-3′), respectively. Using site-directed mutagenesis, we made single and
double mutants of these two putative EBSs in the HO-1-(-4045/+74) promoter construct. Fig. 4A shows a schematic diagram of these mutants. The data are presented as fold induction compared with promoter activity of the wild-type construct in the absence of Ets-2 or LPS. The EBS mutants and wild-type HO-1 promoter constructs were co-transfected with the expression plasmid for Ets-2 or with an equivalent amount of empty vector plus LPS (Fig. 4, B and C, respectively). In the context of the HO-1(-4045/+74) construct, the promoter activity of mEBS1 tended to decrease slightly in the presence of Ets-2, and this decrease was significant in the presence of LPS. The lack of significance in the Ets-2-transfected cells may relate to variability in efficiency and level of Ets-2 expression. However, more impressively, both mEBS2 and mEBS1+2 constructs were markedly decreased compared with the wild-type HO-1(-4045/+74) construct in the presence of Ets-2 or LPS, and in the LPS-stimulated cells, mEBS2 and mEBS1+2 were also significantly decreased compared with mEBS1. Taken together, these results suggest that both EBSs -125 and EBSs -93 contribute to Ets-2 and LPS responsiveness; however, the EBS-93 site is the most critical site responsible for Ets-2 or LPS induction of the HO-1 -4 kb promoter.

To further define the potential role of Ets-2 in the regulation of HO-1, we performed electrophoretic mobility shift assay and microaffinity Ets-2 capture experiments with a radiolabeled probe encoding the EBS2 site at bp -93. The probe was incubated with nuclear extracts from RAW 264.7 cells in the presence or absence of LPS (500 ng/ml) (Fig. 5). Fig. 5A shows that a protein complex bound at the EBS2 site, and the intensity of this complex increased with LPS treatment. To evaluate the specificity of binding, a 100-molar excess of unlabeled identical (Fig. 5A, I, lane 3), mutated (Mt1, lane 4), or non-identical (NI, lane 6; AP-1 binding site) oligonucleotide competitors was incubated with the reaction mixture. These competitor studies revealed that the induced DNA-protein complex binding at the EBS2 site was specific. To determine whether Ets-2 was present in the binding complex, we used a previously described transcription factor microcapture technique (33). The biotinylated EBS2 probe was incubated with nuclear extracts from RAW 264.7 cells treated with vehicle or LPS. Nuclear extracts from cells stimulated with LPS were also incubated in the presence or absence of in vitro translated dominant negative Ets2 (rd-Ets2) and Ets2DBD (rEts2DBD) (Ets-2 and Ets-2DBD). The bound proteins were recovered by magnetic streptavidin beads, washed, and run on a 12% SDS-PAGE gel before performing a Western blot using an Ets2-specific antibody as described under "Experimental Procedures."
Absence of LPS or Ets-2; †, activity. *, /H11001 4045/74 promoter activity. †, /H11001/H11002 74) promoter.

Luciferase activity was plotted as fold induction compared with baseline HO-1-(/H11001 4045/74) promoter activity. *, p < 0.05 versus HO-1-(/H11001 4045/74) promoter activity in the absence of LPS or Ets-2; †, p < 0.05 versus Ets-2 stimulated HO-1-(/H11001 4045/74) promoter activity; #, p < 0.05 versus LPS-treated HO-1-(/H11001 4045/74) promoter activity.

(Dn-Ets-2) prevented this protein from binding at the EBS2 site and that mutation of the EBS2 site prevented binding of wild-type recombinant Ets-2 (rEts-2).

Disruption of DNA Binding or Overexpression of Dominant Negative Ets-2 Blocks Induction of the HO-1 Promoter—We have shown that Ets-2 induces HO-1 transactivation by acting through the EBS2 site at bp -93 and that Ets-2 is capable of binding to this EBS site. To confirm the importance of DNA binding by Ets-2, we generated an expression construct with the DNA binding domain of Ets-2 mutated (Dn-Ets-2). As shown previously (Fig. 1), overexpression of wild-type Ets-2 significantly increased HO-1-(/H11001 4045/74) promoter activity; however, overexpression of Dn-Ets-2 is incapable of trans-activating the HO-1 promoter (Fig. 6). Thus, Ets-2 must bind DNA for this transcription factor to induce the HO-1 promoter (Fig. 4). To examine the role of Ets-2 in regulating HO-1 transactivation by an LPS stimulus, we generated a dominant negative Ets-2 expression plasmid (Dn-Ets-2). The Dn-Ets-2 construct has a DNA binding domain but does not have a transactivation domain; thus, it acts by blocking the binding of functional Ets-2 to the target gene promoter (28, 29). Whereas LPS stimulation markedly induced the HO-1-(/H11001 4045/74) reporter construct, co-expression of Dn-Ets-2 significantly blunted HO-1 promoter transactivation by LPS. These data demonstrate that Ets-2 plays an important role in the transactivation of HO-1 by LPS in mouse macrophages.

PI3K and JNK Regulate Expression of Both Ets-2 and HO-1—Ets-2 is a target of PI3K and JNK signaling pathways (34), and we wanted to determine whether an Ets-2 phosphorylation mutant or an alteration in Ets-2 expression by kinase inhibitors may also affect the level of HO-1 expression. As shown previously, the HO-1-(/H11001 4045/74) reporter construct was significantly induced in RAW 264.7 cells by both LPS and wild-type Ets-2 (Fig. 7A). Conversion of a threonine at position 72 of functional Ets-2 to the target gene promoter (28, 29). Whereas LPS stimulation markedly induced the HO-1-(/H11001 4045/74) promoter activity, * p < 0.05 versus HO-1-(/H11001 4045/74) promoter activity in the absence of LPS or Ets-2; †, p < 0.05 versus Ets-2 stimulated HO-1-(/H11001 4045/74) promoter activity. In B and C, RAW 264.7 cells were treated with inhibitors of PI3K (LY29004), JNK (SP600125), p38 MAPK (SB203580), or ERK1/2 (PD98059) pathways in doses of 5–10 μM, 1 h before LPS stimulation. Total protein was extracted from the cells after 6 (B) or 24 h (C), and Western blot analyses were performed using anti-Ets-2 or anti-HO-1 antibodies, respectively. P-Ets-2 represents the phosphorylated form of Ets-2.

PI3K and JNK inhibition suppresses induction of Ets-2 and HO-1 protein by LPS. A, HO-1-(/H11001 4045/74) promoter construct was co-transfected with empty pCI vector (in the absence or presence of 500 ng/ml LPS), pCI-Ets-2, or pCI-Ets-2T72A (200 ng/well). The cells were harvested 24 h after transfection, and luciferase activity was plotted as fold induction compared with baseline HO-1-(/H11001 4045/74) promoter activity. *, p < 0.05 versus HO-1-(/H11001 4045/74) promoter activity in the absence of LPS or Ets-2; †, p < 0.05 versus LPS or Ets-2 stimulated HO-1-(/H11001 4045/74) promoter activity. B, RAW 264.7 cells were treated with inhibitors of PI3K (LY29004), JNK (SP600125), p38 MAPK (SB203580), or ERK1/2 (PD98059) pathways in doses of 5–10 μM, 1 h before LPS stimulation. Total protein was extracted from the cells after 6 (B) or 24 h (C), and Western blot analyses were performed using anti-Ets-2 or anti-HO-1 antibodies, respectively. P-Ets-2 represents the phosphorylated form of Ets-2.

PI3K and JNK Regulate Expression of Both Ets-2 and HO-1—Ets-2 is a target of PI3K and JNK signaling pathways (34), and we wanted to determine whether an Ets-2 phosphorylation mutant or an alteration in Ets-2 expression by kinase inhibitors may also affect the level of HO-1 expression. As shown previously, the HO-1-(/H11001 4045/74) reporter construct was significantly induced in RAW 264.7 cells by both LPS and wild-type Ets-2 (Fig. 7A). Conversion of a threonine at position 72 of Ets-2 (site of phosphorylation) (31) to an alanine still allowed some transactivation of the HO-1 promoter; however, the overall inducibility was reduced by 54% (Fig. 7A). Moreover, RAW 264.7 cells were treated with inhibitors of PI3K (LY29004), JNK (SP600125), p38 MAPK (SB203580), and ERK1/2 (PD98059) pathways 1 h before LPS stimulation. Total protein was extracted from the RAW 264.7 cells after 6 h (peak Ets-2 induction), and an equal amount of total protein was loaded into each lane. Western blot analysis was performed using an anti-Ets-2 antibody. Inhibition of the JNK pathway blocked formation of the phosphorylated form of Ets-2 (P-Ets-2), whereas inhibition of the PI3K pathway not only blocked formation of phosphorylated Ets-2 but also reduced the overall expression of Ets-2 (Fig. 7B). Inhibitors of p38 MAPK and ERK1/2 had no effect on Ets-2 phosphorylation or expression. Interestingly, experiments were also performed to assess the effect of these inhibitors on HO-1 protein expression by Western blot analysis using an anti-HO-1 antibody. Total protein was extracted from the RAW 264.7 cells after 24 h of LPS stimulation (peak HO-1 induction), and an equal amount of total protein was loaded into each lane. Inhibitors of PI3K and JNK pathways, but not p38 MAPK and ERK1/2 pathways, suppressed HO-1 induction by LPS in macrophages (Fig. 7C).

The most dramatic effect occurred with PI3K inhibition, which dramatically reduced the overall expression of Ets-2. Inhibitors of p38 MAPK and Erk1/2, even given in doses 5-fold higher than those shown in Fig. 7C, did not markedly reduce HO-1 protein levels (data not shown). These data demonstrate the importance of Ets-2 phosphorylation on its ability to transactivate HO-1 and, more importantly, demonstrate that by decreasing overall Ets-2 expression, PI3K can significantly downregulate endogenous HO-1 expression.

DISCUSSION

Ets family proteins play a vital role in the regulation of mammalian immunity (18–20). Family members, such as Ets-1 and Ets-2, participate in the regulation of inflammatory response genes including tumor necrosis factor α, integrins, extracellular proteases, and genes that mediate inflammatory cell survival (35–37). These genes all potentially play a role in the propagation of an inflammatory response. Activation of

Fig. 6. Ets-2 contributes to LPS induction of the HO-1 promoter. HO-1-(/H11001 4045/74) promoter construct was co-transfected with pCI-Ets-2, Ets-2ΔDBD, or dn-Ets-2 (200 ng/well). Empty pCI vector was added as a control and to keep the content of total plasmid DNA constant. After transfection, cells were treated with vehicle or LPS (500 ng/ml) for 24 h and then harvested. Luciferase activity was plotted as fold induction compared with baseline HO-1-(/H11001 4045/74) promoter activity. *, p < 0.05 versus HO-1-(/H11001 4045/74) promoter activity in the absence of LPS or Ets-2; †, p < 0.05 versus Ets-2 stimulated HO-1-(/H11001 4045/74) promoter activity; #, p < 0.05 versus LPS-treated HO-1-(/H11001 4045/74) promoter activity.

Fig. 7. PI3K and JNK inhibition suppresses induction of Ets-2 and HO-1 protein by LPS. A, HO-1-(/H11001 4045/74) promoter construct was co-transfected with empty pCI vector (in the absence or presence of 500 ng/ml LPS), pCI-Ets-2, or pCI-Ets-2T72A (200 ng/well). The cells were harvested 24 h after transfection, and luciferase activity was plotted as fold induction compared with baseline HO-1-(/H11001 4045/74) promoter activity. *, p < 0.05 versus HO-1-(/H11001 4045/74) promoter activity in the absence of LPS or Ets-2; †, p < 0.05 versus LPS or Ets-2 stimulated HO-1-(/H11001 4045/74) promoter activity. In B and C, RAW 264.7 cells were treated with inhibitors of PI3K (LY29004), JNK (SP600125), p38 MAPK (SB203580), or ERK1/2 (PD98059) pathways in doses of 5–10 μM, 1 h before LPS stimulation. Total protein was extracted from the cells after 6 (B) or 24 h (C), and Western blot analyses were performed using anti-Ets-2 or anti-HO-1 antibodies, respectively. P-Ets-2 represents the phosphorylated form of Ets-2.
Ets-2 specifically has been shown to mediate macrophage survival and inflammatory gene expression in a mouse model of chronic inflammation termed the motheaten-viable mutant mouse (34, 37). Whereas the role of factors such as Ets-1 and Ets-2 in a pro-inflammatory response is known, less is known about the ability of these factors to regulate genes that suppress and counterbalance an acute inflammatory response.

HO-1, although acutely induced by inflammatory stimuli, has cytoprotective properties and prevents an exaggerated inflammatory response and oxidative tissue injury in many disease processes, including endotoxia (10, 38). Ets-1 and Ets-2 are both able to induce HO-1 promoter activity (Fig. 1); however, Ets-2 appeared to be a much more potent transactivator of HO-1 in macrophages. Moreover, Ets-2 is induced by the inflammatory mediator LPS at the mRNA and protein level, and this induction preceded the up-regulation of HO-1 (Fig. 2). Ets-1 protein levels were very low at baseline and were not induced by LPS in RAW 264.7 cells (data not shown). Thus, we wanted to further delineate the role of Ets-2 in the up-regulation of HO-1 by LPS in macrophages.

HO-1 is a highly inducible enzyme, with regulation occurring at the level of gene transcription. HO-1 is up-regulated by inflammatory mediators, such as LPS, in macrophages (39). Two enhancer regions located 4 and 10 kb upstream from the transcription start site (40) have been shown to play a role in the induction of HO-1 by LPS in macrophages (24). Both enhancer regions contain multiple AP-1/stress response element binding sites, but because deletion of the –10 kb enhancer does not reduce HO-1 promoter activity in the presence of the –4 kb enhancer (24), our initial focus was the –4 kb enhancer region. Previous studies demonstrated that AP-1 factors (c-Jun/Fos) contribute to LPS responsiveness within this –4 kb enhancer region of the HO-1 promoter (14), whereas Nrf2 regulates induction of HO-1 by stimuli such as heme, heavy metals, and antioxidants (41, 42). Induction of HO-1 (–4045/+74) by Ets-2 was not additionally transactivated by co-transfection with a transcription enhancing plasmid for the AP-1 family member c-Jun (data not shown). These data led us to believe that although AP-1/stress response elements within the –4 kb enhancer are important for HO-1 induction, additional Ets binding sites may contribute to HO-1 induction by endotoxin.

Deletion constructs removing the –4 kb enhancer region did not show a reduction in promoter activity after co-transfection with Ets-2 (Fig. 3A) or exposure to LPS (Fig. 3B) in macrophages. In fact, the deletion mutants down to construct HO-1 (–137/+74) maintained an activity analogous to that of our largest construct, HO-1 (–4045/+74). Further deletion constructs (HO-1 (–117/+74), HO-1 (–66/+74), and HO-1 (–34/+74)) showed a significant reduction in promoter activity when exposed to Ets-2 (Fig. 3A) or LPS (Fig. 3B). Analysis of the promoter sequence revealed two putative EBSs, and mutation of these sites showed that EBS –125 and EBS –93 both contained functional activity. However, EBS –93 was the most critical site responsible for full transactivation of construct HO-1 (–4045/+74) (Fig. 4). Additional studies showed that EBS –93 of the HO-1 promoter binds Ets-2 (Fig. 5) and that mutation of the DNA binding domain of Ets2 entirely prevents transactivation of HO-1 (Fig. 6). However, to truly determine whether Ets-2 contributed to HO-1 transactivation by LPS, we performed studies using a dominant negative form of Ets-2 (dn-Ets-2). Overexpression of dn-Ets-2 along with the HO-1 promoter produced a marked blunting of HO-1 induction by LPS (Fig. 6), providing evidence that Ets-2 contributes to the up-regulation of HO-1 transcription by this potent inflammatory stimulus in macrophages.

Previous studies (15, 43) have demonstrated the importance of kinase signaling pathways, particularly the p38 MAPK pathway, in the anti-inflammatory properties of HO-1 and its product, carbon monoxide. Recent studies have also suggested that PI3K is involved in the regulation of HO-1 expression itself (42, 44). Due to the fact Ets-2 is a target of PI3K and JNK (34) and that phosphorylation and activation of Ets-2 function in macrophage biology and inflammatory gene expression (37), we wanted to determine whether an alteration in Ets-2 phosphorylation and expression by kinase inhibitors may also affect the level of HO-1. Inhibitors of PI3K and JNK pathways decreased the phosphorylated form of Ets-2; however, PI3K inhibition also dramatically decreased overall Ets-2 expression (Fig. 7B).

Interestingly, of the kinase pathways studied, inhibition of PI3K had the most dramatic effect on preventing HO-1 induction by LPS in macrophages (Fig. 7C). These data suggest that the effect of Ets-2 on HO-1 expression goes beyond a phosphorylation event. Other kinase inhibitors, including p38 MAPK and ERK1/2, had no significant effect on Ets-2 or HO-1 induction by LPS in macrophages. Taken together, these data support our hypothesis that Ets-2 plays an important role in the induction of HO-1 by LPS in macrophages and that kinase pathways, particularly PI3K, may be involved in the regulation of Ets-2 that contributes to HO-1 expression.

Ets-2 is a transcription factor known to be involved in the immune response, regulating genes that play a vital role in the propagation of an inflammatory response (35–37). Our results reveal for the first time that Ets-2 contributes to the transcriptional regulation of HO-1, a cytoprotective gene with anti-inflammatory and anti-oxidant properties. Ets-2 is a potent transactivator of HO-1, and Ets-2 itself is induced by the potent inflammatory stimulus LPS before HO-1 induction. Surprisingly, the effect of Ets-2 (and also LPS) on HO-1 up-regulation appears to be functioning through Ets binding sites in the downstream HO-1 promoter, with particular importance at bp –93. Moreover, using a dominant negative form of Ets-2, we were able to suppress HO-1 promoter induction by LPS in macrophages, and kinase inhibitors (PI3K more than JNK) that reduce Ets-2 expression markedly decrease endogenous HO-1 expression. These data provide evidence that Ets-2 is an important regulator of HO-1 expression during LPS stimulation and that up-regulation of this inflammation-responsive, cytoprotective gene may provide a mechanism by which Ets-2 counterbalances and protects against an overwhelming inflammatory response.

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Role of Ets-2 in the Regulation of Heme Oxygenase-1 by Endotoxin
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