Regulation of heme oxygenase-1 gene by peptidoglycan involves the interaction of Elk-1 and C/EBPα to increase expression

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Heme oxygenase (HO) is the rate-limiting enzyme in heme degradation that produces equimolar concentrations of carbon monoxide (CO), biliverdin, and ferrous iron (38, 39). Biliverdin is subsequently converted into bilirubin by biliverdin reductase (33, 35). Three different isoforms of HO have been described, and HO-1 is a very inducible isoform in response to many pathophysiological stimuli, including proinflammatory mediators (2). HO-1 and its products, CO and bilirubin, have anti-inflammatory and antioxidant properties, respectively (1, 24, 31, 32). These properties are critical during the inflammatory response; Gram-positive; macrophage; gene regulation; transcription

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such as Ets-2 and, to a lesser extent, Ets-1 contribute to the LPS induction of HO-1 in macrophages via an Ets binding site, EBS2, located in the proximal promoter of mouse HO-1. Moreover, LPS-induced downregulation of the repressor Elk-3, which belongs to the ternary complex factors (TCF) subfamily of Ets factors, facilitates HO-1 induction in macrophages via a different Ets binding site, EBS1, just upstream of EBS2 (12). Because of the importance of these two EBS in the proximal HO-1 promoter during LPS stimulation, we also wanted to elucidate the potential role of Ets family members and their associated binding sites in driving HO-1 transcription by PGN of a Gram-positive bacterial source.

MATERIALS AND METHODS

Cell culture and reagents. Murine macrophages (RAW 264.7) (15) and fibroblasts (NIH/3T3) were grown according to the recommendations of American Type Culture Collection. PGN from S. aureus was purchased from Sigma-Aldrich. PGN is dissolved in 50% DMSO and sonicated (Model 500 Digital Sonic Dismembrator; Fisher Scientific) before use.

Mouse model of a systemic Gram-positive stimulus. HO-1 (−/−) mice were generated as described previously (47). Female mice were used for these studies, and all mice were on a pure BALB/c genetic background. HO-1 (−/−) and HO-1 (+/+) mice were injected intraperitoneally with PGN (20 mg/kg). Survival was assessed every 8 h for 7 days. Spleens, kidneys, and lungs were harvested at baseline and after 6, 12, and 24 h of PGN stimulation. All PGN experiments in mice were performed in accordance with National Institutes of Health (NIH) guidelines and were approved by Harvard Medical Area standing committee on animals.

RNA isolation and Northern blot and real-time PCR analyses. Extraction of total RNA from cultured cells and mouse tissues was performed using the RNeasy Mini RNA isolation kit (Qiagen). Northern blot analysis was performed as described previously (11), using a random-primed, [α-32P]dCTP-labeled HO-1 cDNA probe. To correct for the differences in RNA loading, blots were subsequently hybridized to a [32P]-labeled oligonucleotide probe complementary to 18S rRNA. Radioactivity was quantitated on a Phosphorimager using University School of Medicine, New York, NY). mEBS1 and mEBS2 pcDNA3-Elk-1 was a gift from Dr. F. M. Stanley (New York Oettgen (Beth Israel Deaconess Medical Center, Boston, MA), and Ets-2, pCI-Elk-3, and pCI-NERF2 were a generous gift from Dr. Peter (12).

Site-directed mutagenesis. The fragments into the pGL2-Basic Vector (Promega) as previously described (11, 12). pCI-Elk-1 plasmid using Pfu polymerase (Stratagene). Individual plasmids were sequenced to verify incorporation of the C/EBP site mutation. Deletion of Elk-1 DNA binding domain (Elk-1 ΔDBD), from the 2nd to the 93rd amino acid, was performed by site-directed mutagenesis of the Elk-1 plasmid, the same method as described above. In brief, PCR primers encoding upstream and downstream sequences of the deletion region were generated (5′-CTCGAGGAACATTTCTTCAGGAGTCTCGAGAG-3′ and 5′-cagcttcaaggagcttttggtg-3′). Single underline indicates 1st amino acid sequence, and double underline indicates 94th amino acid sequence.

Transient transfection and reporter activity assays. Transient transfection assays were performed using FuGENE 6 transfection reagent (Roche Applied Science) as described previously (11, 12). The HO-1 promoter-reporter plasmid (200 ng/well) and the indicated amounts of Ets factors, C/EBP factors, and empty vector were cotransfected into murine macrophages. For assays in RAW 264.7 cells, 3 × 105 cells/well were plated in triplicate on 6-well plates and incubated for 24 h. Twenty-four hours later, vehicle or PGN (1 μg/ml) was administered. The cells were harvested for luciferase activity using the Luciferase Assay System (Promega) 24 h after treatment. Luciferase activity was measured in a Wallace Victor3 1420 multilabel counter (PerkinElmer).

Silencing of C/EBPx in mouse macrophages. Short hairpin RNA (shRNA) plasmids for C/EBPx (Clone NM_007687.1-89861c1; Sigma-Aldrich) and vector control (pLKO.1-puro) were transfected into RAW 264.7 cells, and stable clones were selected using puromycin (5 μg/ml) resistance. Silencing of C/EBPx was confirmed by Western blot analysis. Clones silencing C/EBPx and control vector were subsequently transfected with a HO-1 promoter-reporter construct and an expression plasmid for Elk-1 or stimulated with PGN as described for the reporter activity assays.

EMSAs. EMSAs were performed as described previously (8), using nuclear extracts from RAW 264.7 cells stimulated with vehicle or PGN (1 μg/ml) for 1 h. Double-stranded oligonucleotide probes encoding region −98 to −74 of the HO-1 5′-flanking sequence, C/EBP probe (5′-GGGCTGGATGTGTCAGACGAGCA-3′; the C/EBP binding site is underlined) and mC/EBP probe (5′-GGGCTG- GATTTCCACACAGCCAGG-3′), were used. The probes were end-labeled with [α-32P]dATP using T4 polynucleotide kinase (New England Biolabs). Labeled DNA (~50,000 counts/min) was used in each binding reaction. In cold competition assays, a 100-fold molar excess of unlabeled double-stranded oligonucleotides as indicated were added to the binding reactions. For mobility shift experiments, 1 μg of C/EBP probe polyclonal antibody (Santa Cruz Biotechnology) was added to the binding reaction and incubated at room temperature for 2 h before the addition of radioactive probe.

Total protein isolation and Western blot analysis. Protein isolation and Western blotting were performed as described previously (11). Rabbit polyclonal anti-HO-1 (Stressgen Biotechnologies) antibody was diluted to 1:2,000 before use. Rabbit polyclonal anti-Elk-1 and anti-C/EBP antibodies (Santa Cruz Biotechnology) were diluted to 1:1,000 before use. The blots were exposed to X-ray film and evaluated with NIH ImageJ software.

Coimmunoprecipitation assays. Protein G Plus/Protein A Agarose (Calbiochem) beads were washed by PBS and resuspended with dilution buffer (PBS + 1 mg/ml BSA). Elk-1 antibody or C/EBP antibody, 5 μg in dilution buffer, was added at 1:1 ratio to the beads. Dimethyl sulfoxide (1 mg/ml) was added to prevent IgG binding to the beads. The beads were agitated for 30 min at room temperature and then washed three times with buffer (0.2 M triethanolamine in PBS) before quenching the cross-linking with 50 mM ethanolamine in PBS. Finally, unlabeled antibody was washed out with 1 M glycine, pH 3, and the cross-linked beads were reconstituted in PBS.

RAW 264.7 cells were treated with vehicle or PGN (1 μg/ml). Cells were harvested 1 h after treatment, and the nuclear protein extract was isolated with lysis buffer (20 mM Tris-HCl, pH 7.5, 2% sucrose, 420 mM NaCl, 5 mM dithiothreitol, 2 mM MgCl2, 0.2 mM
EDTA, 1× protease inhibitor mixture (Roche Applied Science)]. Lysate proteins (400 μg) were incubated with the antibody-cross-linked beads at 4°C overnight. The beads were then washed four times with a cold buffer (20 mM Tris-HCl, pH 7.2, 1 mM EDTA, 0.1% Triton X-100, 150 mM NaCl, 1 mg/ml BSA, and 1× protease inhibitor mixture), and bounded proteins were separated via gradient SDS-PAGE gel (4–20%) and transferred to nitrocellulose membranes for Western blot analysis (48).

Statistics. Comparisons between groups where indicated were made by factorial ANOVA followed by Fisher protected least significant differences test. Comparisons of mortality were made by analyzing Kaplan-Meier survival curves and then log-rank test to assess for differences in survival. Statistical significance is accepted at P < 0.05.

RESULTS

Endogenous HO-1 is induced by PGN, and absence of HO-1 increases mortality during PGN-induced sepsis-like syndrome. To determine whether the expression of HO-1 is regulated by PGN in vivo, we administered PGN (20 mg/kg ip) to wild-type mice and assessed HO-1 protein expression in spleen, kidney, and lung tissue after 0, 6, 12, and 24 h. Western blot analysis revealed increased HO-1 protein expression at 6 h, and protein levels of HO-1 remained elevated throughout 24 h in all three organs (Fig. 1A). HO-1 mRNA levels had a similar pattern (Fig. 1B). Next, to assess the functional significance of this HO-1 expression, we assessed survival of HO-1+/+ and HO-1−/− mice after administration of PGN (20 mg/kg ip). HO-1−/− mice had a very high mortality rate when exposed to PGN, reaching 80% by day 2. In contrast, this dose of PGN was not lethal in HO-1+/+ mice (Fig. 1C). These data suggest that expression of endogenous HO-1 plays a vital role in protecting mice from the lethal consequences of systemic PGN administration.

Induction of HO-1 by PGN in macrophages in vitro. Macrophages are important cellular mediators during sepsis and endotoxemia (21), and these cells have also been widely used in the study of PGN response (45). Thus we wanted to determine whether PGN would induce HO-1 expression in macrophages. We found that PGN increased HO-1 protein and mRNA levels in a dose-dependent manner (with a dose as low as 0.5 μg/ml and up to 5 μg/ml, data not shown) in mouse macrophages (RAW 264.7 cells). A dose of 1 μg/ml PGN was chosen for further experiments. To determine the temporal expression pattern of HO-1 during PGN exposure, we administered 1 μg/ml PGN to RAW 264.7 cells and harvested protein and total RNA at various time points, including 0, 0.5, 1, 2, 6, 12, and 24 h. Similar to the in vivo analysis of HO-1 in tissues, Western blot analysis revealed that protein levels of HO-1 increased by 6 h and remained elevated throughout 24 h. HO-1 induction was 2.1-, 2.8-, and 4.5-fold at 6, 12, and 24 h, respectively, compared with 0 h (P < 0.05; Fig. 2A). By Northern blot analysis, HO-1 mRNA began to increase slightly by 2 h, but the levels of mRNA were significantly increased by 6, 12, and 24 h, with 11.0-, 14.5-, and 18.9-fold increases, respectively, compared with 0 h (P < 0.05; Fig. 2B). We also stimulated another cell type, fibroblasts (NIH/3T3 cells), with a comparable dose of PGN (1 μg/ml) and found no significant induction of HO-1 protein or mRNA (Fig. 3) in contrast to macrophages. Other investigators have shown in fibroblasts (from the synovium of rheumatoid arthritis patients, thus already in an inflammatory environment) that much higher doses of PGN (a minimum of 10 μg/ml and up to 30–100 μg/ml) are required to further induce the immune mediator IL-6 (9). These data suggest that HO-1 induction in macrophages is more sensitive to PGN stimulation, as demonstrated by the HO-1 response in macrophages compared with fibroblasts.
PGN response element(s) are between −117 and −66 bp of the HO-1 promoter. HO-1 is well-known to be regulated at the level of gene transcription (2). Thus, to determine the location of the PGN response element(s) in the HO-1 promoter, we made deletion mutants of construct HO-1 (4,045/74) and transiently transfected these constructs into RAW 264.7 cells exposed to vehicle or PGN (1 μg/ml). In the absence of PGN (Fig. 3, open bars), promoter activity began to decrease in construct HO-1 (66/74), but a significant and marked drop in activity did not occur until HO-1 (35/74) compared with all other constructs ($P < 0.05$; Fig. 4). As expected, HO-1 promoter activity (−4,045/+74) was significantly induced by PGN (Fig. 4, filled bars). However, different from the vehicle experiments, the most dramatic decrease in PGN-induced promoter activity ($P < 0.05$; Fig. 4) occurred with deletion to construct HO-1 (−66/+74). These results suggest that critical cis-acting element(s) responsible for HO-1 induction by PGN in macrophages are located between −117 and −66 bp of the HO-1 promoter.

Elk-1 is an activator of the HO-1 promoter. We (11) have previously shown that the EBS2 site (−93 bp) is important for induction of HO-1 by LPS stimulation. Thus we hypothesized that Ets transcription factors may also be important for regulation of HO-1 by PGN. To determine which Ets factors may contribute to HO-1 induction by PGN, we examined the ability of a panel of Ets factors to transactivate the HO-1 promoter. RAW 264.7 cells were transiently cotransfected with HO-1 (−4,045/+74) and expression plasmids for NERF2, Elk-1, Elk-3, Ets-1, and Ets-2 in the presence of vehicle or PGN (1 μg/ml). In the absence of PGN (Fig. 5, open bars), Elk-1 and Ets-2 were the most potent inducers of the HO-1 promoter ($P < 0.05$; Fig. 5A). To further evaluate the induction of HO-1 promoter activity by Elk-1 alone, we cotransfected HO-1 (−4,045/+74) with increasing amounts of the Elk-1 expression plasmid and demonstrated an increase in HO-1 promoter activity in a dose-dependent manner (up to 4.8-fold with 400 ng/well, data not shown). In the presence of PGN (Fig. 5, filled bars), Elk-1 and Ets-2 further increased HO-1 promoter activity by 12.2- and 7.5-fold, respectively, compared with baseline pCI control ($P < 0.05$; Fig. 5A). To localize the Elk-1-
responsive element(s) in the HO-1 promoter, we transiently cotransfected deletion mutants of the HO-1 promoter and an Elk-1 expression plasmid into RAW 264.7 cells in the presence or absence of PGN (1 μg/ml). We found that, analogous to PGN (Fig. 4), induction of HO-1 promoter activity by Elk-1 alone or Elk-1 + PGN significantly decreased at deletion mutant HO-1 (−66/+74) compared with constructs containing additional upstream regions of the promoter (−117 through −4,045 bp; Fig. 5B). These data verify that Elk-1 is a transactivator of the HO-1 promoter and that the region of the HO-1 promoter responsible for Elk-1 transactivation lies in the same location as the element(s) responsible for induction of the HO-1 promoter by PGN (between −117 and −66 bp).

**EBS are not responsible for HO-1 induction by Elk-1 and PGN.** To identify the cis-acting element(s) responsible for Elk-1 and PGN transactivation of the HO-1 promoter, we mutated two EBS in promoter construct HO-1 (−295/+74), termed mEBS1 and mEBS2 (11). The wild-type, mEBS1, or mEBS2 HO-1 (−295/+74) promoter constructs were cotransfected into RAW 264.7 cells with Elk-1 expression plasmid and exposed to either vehicle or PGN (1 μg/ml). The promoter activity in the presence of Elk-1 or Elk-1 + PGN was not different between wild-type, mEBS1, and mEBS2 HO-1 promoter constructs (Fig. 6A). These data suggest that Elk-1 may activate the HO-1 promoter independent of binding to EBS1 or EBS2. To test this hypothesis, we generated an Elk-1 mutant expression plasmid with Elk-1 ΔDBD. The empty pCI vector, wild-type Elk-1, or Elk-1 ΔDBD expression plasmids were cotransfected with HO-1 (−295/+74) promoter into RAW 264.7 cells and exposed to vehicle or PGN (1 μg/ml). Both wild-type Elk-1 and Elk-1 ΔDBD were capable of increasing HO-1 promoter activity compared with empty pCI vector. Furthermore, wild-type Elk-1 and Elk-1 ΔDBD were able to enhance transactivation of the HO-1 promoter in the presence of PGN (Fig. 6B). These data suggest that DNA binding is not required for Elk-1 to transactivate the HO-1 promoter and support our data showing no difference in activity of wild-type and mEBS promoter constructs exposed to Elk-1 and PGN.

C/EBPα together with Elk-1 induce HO-1 promoter activity. A cis-acting element just downstream of EBS2 in the HO-1 promoter, C/EBP binding site, was considered a potential mediator of this PGN response because C/EBP proteins are transcription factors known to be activated by oxidative stress (10). We thus mutated the C/EBP binding site starting at −90 bp (designated mC/EBP) in the HO-1 (−295/+74) construct. The wild-type and mC/EBP HO-1 (−295/+74) promoter constructs were cotransfected into RAW 264.7 cells with Elk-1 expression plasmid in the presence or absence of PGN (1 μg/ml). The mC/EBP HO-1 promoter activity in the presence of Elk-1 alone or Elk-1 + PGN was

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**Fig. 4.** HO-1 promoter has PGN-responsive element(s) downstream of −117 bp. Cells were transiently transfected with deletion mutants of the HO-1 promoter (200 ng/well). After the transfection, cells were treated with vehicle (open bars) or PGN (1 μg/ml; filled bars) for 24 h and then harvested for luciferase assay. HO-1 promoter activity was calculated as fold induction compared with the activity of construct HO-1 (−4,045/+74) exposed to vehicle. Four independent experiments were performed in triplicate. *P < 0.05 vs. all other constructs exposed to vehicle.

**Fig. 5.** Ets factors transactivate the HO-1 promoter, and Elk-1-responsive element(s) are located in the region analogous to PGN-responsive element(s). A: cells were transiently cotransfected with deletion mutants of the HO-1 promoter (−4,045/+74) promoter construct (200 ng/well) and Ets expression plasmids (pCI-NERF2, pCI-Elk-1, pCI-Elk-3, pCI-Ets1, and pCI-Ets-2) or empty pCI vector (400 ng/well). After the transfection, cells were treated with vehicle (open bars) or PGN (1 μg/ml; filled bars) for 24 h and then harvested for luciferase assay. Four independent experiments were performed in triplicate. *P < 0.05 vs. pCI control with PGN. †P < 0.05 vs. pCI control with vehicle. B: cells were transiently cotransfected with deletion mutants of the HO-1 promoter (200 ng/well) and pCI-Ets1 expression plasmid (400 ng/well). After the transfection, cells were treated with vehicle (open bars) or PGN (1 μg/ml; filled bars) for 24 h and then harvested for luciferase assay. Four independent experiments were performed in triplicate. *P < 0.05 vs. activity of HO-1 (−4,045/+74), (−1,867/+74), (−295/+74), and (−117/+74) promoter constructs stimulated with PGN. †P < 0.05 vs. HO-1 (−4,045/+74) promoter construct with vehicle.
activity (5.5-fold), which was significantly greater than C/EBP binding site.

moter by Elk-1 and PGN and support the hypothesis that binding site is important for activation of the HO-1 promoter (Fig. 7A). These data suggest that the C/EBP promoter (Fig. 7C, lane 3 compared with lane 2). To evaluate the specificity of binding, a 100-fold molar excess of unlabeled identical (Fig. 7C, lane 4) or mutated (Fig. 7C, lane 5) oligonucleotide competitors was incubated with the reaction mixture. The identical competitor completely disrupted the DNA-protein complexes, and this disruption did not occur with the mC/EBP competitor, suggesting specific binding of these complexes to the C/EBP probe. To determine whether C/EBPα was present within the binding complex, C/EBPα antibody (Fig. 7C, lane 6) or rabbit IgG (control; Fig. 7C, lane 7) was incubated with the reaction mixture. This revealed that the C/EBPα antibody disrupted the 3rd DNA-protein complex (Fig. 7C, "<") and supershifted the complex (Fig. 7C, arrow), whereas rabbit IgG had no effect. These results indicated that the binding of C/EBPα to the C/EBP binding site on the HO-1 promoter is specific and increased by PGN.

PGN induces C/EBPα binding to the HO-1 promoter. To further understand the role of C/EBPα in the induction of HO-1 by PGN, we performed an EMSA with a radiolabeled probe encoding the C/EBP binding site at −89 to −82 bp of the HO-1 promoter. The probe was incubated with nuclear extracts from RAW 264.7 cells in the presence or absence of PGN (1 μg/ml). Four inducible protein complexes bounded at the C/EBP binding site, and the intensity of these complexes increased after PGN treatment (Fig. 7C, lane 3 compared with lane 2). To evaluate the specificity of binding, a 100-fold molar excess of unlabeled identical (Fig. 7C, lane 4) or mutated (Fig. 7C, lane 5) oligonucleotide competitors was incubated with the reaction mixture. The identical competitor completely disrupted the DNA-protein complexes, and this disruption did not occur with the mC/EBP competitor, suggesting specific binding of these complexes to the C/EBP probe. To determine whether C/EBPα was present within the binding complex, C/EBPα antibody (Fig. 7C, lane 6) or rabbit IgG (control; Fig. 7C, lane 7) was incubated with the reaction mixture. This revealed that the C/EBPα antibody disrupted the 3rd DNA-protein complex (Fig. 7C, "<") and supershifted the complex (Fig. 7C, arrow), whereas rabbit IgG had no effect. These results indicated that the binding of C/EBPα to the C/EBP binding site on the HO-1 promoter is specific and increased by PGN.

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cells. Western blot analysis revealed that shRNA decreased C/EBPα expression by 78.3 ± 2.1% in mouse macrophages compared with vector control cells (Fig. 9A). Next, we transfected C/EBPα shRNA cells and control cells with construct HO-1 (~295/74) in the presence or absence of an expression plasmid for Elk-1. Compared with vector control cells, which showed an induction of the HO-1 promoter to a similar degree as wild-type cells (Fig. 9A), Elk-1 did not increase HO-1 promoter activity in C/EBPα-silenced cells (Fig. 9B). In addition, C/EBPα shRNA cells showed no increase in HO-1 promoter activity when exposed to PGN (1 μg/ml), in contrast with the induction seen in vector control cells (Fig. 9C). Taken together, these data demonstrate the importance of C/EBPα for HO-1 transactivation by Elk-1 and PGN. Figure 9D provides a schema of how the interaction of Elk-1 and C/EBPα, through

Fig. 7. CCAAT/enhancer-binding protein-α (C/EBPα) induces HO-1 promoter activity together with Elk-1 and binds to a C/EBP binding site. A: cells were transiently cotransfected with HO-1 (~295/74) promoter construct containing WT (HO-1 WT) or mutated C/EBP binding site at ~90 (HO-1 mC/EBP; 200 ng/well) and pCI-Elk-1 expression plasmid (400 ng/well). After the transfection, cells were treated with vehicle (open bars) or PGN (1 μg/ml; filled bars) for 24 h and then harvested for luciferase assay. Four independent experiments were performed in triplicate. *P < 0.05 compared with HO-1 WT construct stimulated with PGN. †P < 0.05 vs. HO-1 WT with vehicle. B: cells were transiently cotransfected with HO-1 (~295/74) promoter construct (200 ng/well) and either empty pCI vector (open bars; 400 ng/well) or pCI-Elk-1 expression plasmid (filled bars; 400 ng/well). Both groups were also cotransfected with empty pCI vector, pCI-C/EBPα, pCI-C/EBPβ, or pCI-C/EBPβ expression plasmids (400 ng/well). After the transfections, cells were harvested at 48 h for luciferase assay. Four independent experiments were performed in triplicate. *P < 0.001 vs. pCI control with Elk-1, †P < 0.05 vs. pCI control without Elk-1. §P < 0.05 vs. pCI-C/EBPα without Elk-1. C: EMSA was performed using nuclear proteins harvested from cells 1 h following treatment with vehicle or PGN (1 μg/ml). Nuclear proteins were first incubated with cold C/EBP site probe (C), cold mutated C/EBP site probe (mC), anti-C/EBPα antibody (ab), or control rabbit IgG as indicated. A radiolabeled C/EBP site probe (~98/74) was then added into the reaction mixture and subjected to electrophoresis. Details are described in MATERIALS AND METHODS. ††, Specific band identified by antibody. Experiments in A and B were performed 2 independent times.

Fig. 8. PGN induces C/EBPα and Elk-1 interaction. A: coimmunoprecipitation (co-IP) was performed using nuclear protein extract harvested from cells 1 h following treatment with vehicle or PGN (1 μg/ml). Cellular proteins were incubated with Elk-1 antibody-cross-linked protein A/G beads, rabbit IgG-cross-linked protein A/G beads, or protein A/G beads alone. The bounded proteins were separated with SDS-PAGE. Western blot analysis of IP-bounded proteins (lanes 1–4) and pre-IP cellular proteins (lanes 5 and 6) were performed using C/EBPα antibody. B: co-IP was performed as described above. Cellular proteins were incubated with C/EBPα antibody-cross-linked protein A/G beads, rabbit IgG-cross-linked protein A/G beads, or protein A/G beads alone. The bounded proteins were separated with SDS-PAGE. Western blot analysis of IP-bounded proteins (lanes 1–4) and pre-IP cellular proteins (lanes 5 and 6) were performed using Elk-1 antibody. Details are described in MATERIALS AND METHODS. *P < 0.05 vs. pCI-C/EBPα without Elk-1. Fig. 9. C/EBPα binds to a C/EBP binding site and increases HO-1 promoter activity in the presence of Elk-1 and PGN. A: cells were transiently cotransfected with HO-1 (~295/74) promoter construct containing WT (HO-1 WT) or mutated C/EBP binding site at ~90 (HO-1 mC/EBP; 200 ng/well) and pCI-Elk-1 expression plasmid (400 ng/well). After the transfection, cells were treated with vehicle (open bars) or PGN (1 μg/ml; filled bars) for 24 h and then harvested for luciferase assay. Four independent experiments were performed in triplicate. *P < 0.05 compared with HO-1 WT construct stimulated with PGN. †P < 0.05 vs. HO-1 WT with vehicle. B: cells were transiently cotransfected with HO-1 (~295/74) promoter construct (200 ng/well) and either empty pCI vector (open bars; 400 ng/well) or pCI-Elk-1 expression plasmid (filled bars; 400 ng/well). Both groups were also cotransfected with empty pCI vector, pCI-C/EBPα, pCI-C/EBPβ, or pCI-C/EBPβ expression plasmids (400 ng/well). After the transfections, cells were harvested at 48 h for luciferase assay. Four independent experiments were performed in triplicate. *P < 0.001 vs. pCI control with Elk-1, †P < 0.05 vs. pCI control without Elk-1. §P < 0.05 vs. pCI-C/EBPα without Elk-1. C: EMSA was performed using nuclear proteins harvested from cells 1 h following treatment with vehicle or PGN (1 μg/ml). Nuclear proteins were first incubated with a reaction mixture containing cold C/EBP site probe (C), cold mutated C/EBP site probe (mC), anti-C/EBPα antibody (ab), or control rabbit IgG as indicated. A radiolabeled C/EBP site probe (~98/74) was then added into the reaction mixture and subjected to electrophoresis. Details are described in MATERIALS AND METHODS. ††, Specific band identified by antibody. Experiments in A and B were performed 2 independent times.
focused on the use of LPS from Gram-negative bacteria (7). This was, in part, due to the fact that sepsis was considered by many to be near-synonymous with Gram-negative endotoxemia. However, recently it has become more obvious that the concept of sepsis can arise from microbial infections of different sources (4). For example, Gram-positive bacteria now account for up to 50% of all cases of sepsis (4, 27). Thus we decided to investigate a Gram-positive mediator and its role in a systemic inflammatory response. The major component of the cell wall of Gram-positive bacterial is PGN (27) along with LTA (36). Toll-like receptor (TLR)-2 is a known receptor of LTA, and recently it has been shown that LTA induces HO-1 through the TLR2/MyD88/TRAF6 pathway, causing a Nrf2-dependent transactivation of the HO-1 promoter in tracheal smooth muscle cells (23). TLR2 was also initially identified as the receptor of PGN (19, 34), however, this concept has been challenged using more highly purified preparations of PGN (27, 41). Although the role of TLR2 in PGN signaling may be in question, specific PGN moieties are detected by the cytoplasmic proteins of the NOD family, including NOD2. Since HO-1 plays a protective role in the host defense response to Gram-positive bacteria (13), and this response occurs, in part, through the expression of NOD2, we focused our study on the regulation of the HO-1 gene by PGN from *S. aureus* in inflammatory cells.

The biological significance of HO-1 expression during *S. aureus* PGN exposure was demonstrated by the fact that only 20% of HO-1−/− mice survived this Gram-positive inflammatory stimulus, whereas HO-1+/+ mice showed no lethality (100% survival) to PGN administration (20 mg/kg ip; Fig. 1C). Systemic administration of PGN is a pure inflammatory stimulus, suggesting that the anti-inflammatory and antioxidant properties of HO-1, and its products of heme catabolism, play a cytoprotective role (1, 24, 31, 32). However, in the setting of a Gram-positive microbial infection, we (13) showed previously that HO-1-derived CO is also able to modulate the function of inflammatory cells, promoting increased phagocytosis of bacteria. Thus we believe the beneficial effects of HO-1 during a systemic inflammatory response are multifactorial. Moreover, although mortality is increased in the absence of HO-1, it is clear that increased endogenous expression of HO-1 is not necessarily sufficient to completely protect against death due to endotoxemia or sepsis. To confirm this concept, we showed that overexpression of HO-1, or administration of CO, rescued mice deficient in HO-1 or improved outcome during endotoxemia (37) and sepsis (13) in wild-type mice.

Since expression of HO-1 is critical for survival during systemic administration of PGN, we further explored the mechanism responsible for HO-1 gene regulation by PGN. HO-1 was inducible at both the protein and mRNA level in vivo in tissue (Fig. 1) and in vitro in macrophages (Fig. 2), and this regulation occurred at the level of gene transcription as HO-1 promoter constructs were induced by PGN (Fig. 4). The region of the HO-1 promoter responsible for PGN induction was between −117 and −66 bp (Fig. 4), a region not known to be regulated by Nrf2 (a transcription factor involved in HO-1 regulation by LTA) (23). In fact, this region of the HO-1 promoter is more similar to the LPS-responsive region (11, 12), containing one of the downstream EBS (located at −93 bp, EBS2). However, different from LPS stimulation, expression of Elk-1 in conjunction with PGN (more than Ets-2 or Ets-1)
was the most prominent inducer of HO-1 (Fig. 5A). Elk-1 is a member of the TCF subfamily of Ets transcription factors (20, 43, 46), and previously it has been shown that Elk-1 contributes to the generation of proinflammatory cytokines (such as tumor necrosis factor-α) by PGN in macrophages (45).

Interestingly, the induction of HO-1 did not depend on the direct interaction of Elk-1 with DNA (Fig. 6). A prior study reported the ability of C/EBPβ and Elk-1 to interact and drive gene transcription of the immediate early gene family member c-fos (18). Because of this fact, and the presence of a C/EBP binding site at −90 bp (between −117 and −66 bp and in close proximity to EBS2), we further explored the potential role of C/EBP factors in conjunction with Elk-1 to drive PGN-induced HO-1 promoter activity. Different from disruption of EBS2 and EBS1, mutation of the C/EBP binding site significantly reduced HO-1 promoter activity by Elk-1 in the presence or absence of PGN (Fig. 7A). Analysis of C/EBP factors demonstrated that the most potent transactivator of HO-1 was C/EBPα, and this response was further increased by coexpression with Elk-1 (Fig. 7B). In addition, stimulation of macrophages with PGN increased the co-IP of C/EBPα and Elk-1 (Fig. 8), establishing the interaction of endogenous Elk-1 and C/EBPα in the cell. The activity and/or expression of C/EBP factors are known to be regulated by inflammatory stimuli, including LPS, with induction of C/EBPβ and C/EBPδ, and reduction of C/EBPα, in a number of cell types (29, 30). However, in our study, C/EBPα and its interaction with Elk-1 was increased by PGN, and silencing C/EBPα in macrophages completely abrogated induction of HO-1 promoter by either Elk-1 or PGN (Fig. 9, B and C). These data provide another means by which a C/EBP factor may modulate an inflammatory response, by the regulation of a gene with known anti-inflammatory properties.

Taking together the HO-1 promoter evaluation, the protein-DNA binding studies, and the protein-protein interaction studies, we believe Elk-1 interacts with C/EBPα, and this complex binds to the C/EBP site of the HO-1 promoter, contributing to its transactivation by PGN (Fig. 9D, illustration). In reviewing the literature regarding the transcriptional regulation of HO-1, Alam and Cook (2) hypothesized a similar scenario regarding the regulation of HO-1 by NF-κB. Studies using inhibitors of NF-κB have implicated a role for this transcription factor in the regulation of HO-1, even with the absence of a clearly identified functional binding site in the promoter. Thus the authors (2) hypothesized that a transcription factor may have its effects independent of direct DNA binding, but rather through its association with other DNA-binding proteins within a complex on the HO-1 promoter. In the present study, we demonstrate this concept, showing that Elk-1 drives HO-1 transcription without evidence of direct binding to DNA, but rather by interacting with another DNA-binding protein, C/EBPα. Our study provides further insight into the regulation of HO-1 by an inflammatory mediator of a Gram-positive bacterial source.

REFERENCES


