Negative Regulation of Human Heme Oxygenase in Microvessel Endothelial Cells by Dexamethasone (44443)

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Abstract. **Heme oxygenase-1 (HO-1) is a stress protein, and its induction has been suggested to participate in defense mechanisms against agents that promote oxidative injury such as endotoxins and heme. We have shown that the inflammatory cytokines, interleukin-6 (IL-6) and heme-induced HO-1 gene expression, were suppressed by dexamethasone (Dex) in a sustained manner. We examined the mechanism by which the anti-inflammatory agent, Dex, inhibits IL-6 and heme-induced HO-1 expression in rabbit coronary endothelial cells. Endothelial cells treated with heme (10 μ*M*) and IL-6 (25 ng/ml), increased HO-1 mRNA 15- and 60-fold, respectively. The activity of HO was increased 3-fold after such treatment. Although Dex failed to inhibit heme-mediated HO-1 mRNA and HO activity, it was able to reverse IL-6–stimulated HO activity. Several human HO-1 promoter-drive chloramphenicol acetyltransferase (CAT) constructs were examined to analyze IL-6 and Dex-mediated modulation of the HO-1 gene in endothelial cells. CAT assays revealed that the HO-1 promoter region between −180 and −1500 might contain a Dex-mediated negative regulator. Gel mobility shift assays using nuclear extracts from IL-6–treated endothelial cells showed a binding to the synthetic 21 base pairs of the HO-1 sequence that contains the putative STAT3 sequence. STAT3-specific probe inhibited nuclear binding protein to the putative HO-1-STAT3 sequence. This suggests that IL-6 induction of human HO-1 is mediated *via* the JAK-STAT pathway and that Dex inhibition of gene expression is carried out by activation of a transcriptional protein in competition with the STAT3 binding site.** [P.S.E.B.M. 1999, Vol 222]

A variety of oxidative stress–inducing agents, such as metals, bacterial infection, and hemoglobin, have been implicated in the pathogenesis of the inflammatory process. The cellular response to such agents involves the production of a number of soluble mediators, including acute phase proteins, eicosanoids, and various cytokines. The rate-limiting enzyme in heme catabolism, heme oxygenase (HO), is a stres protein, and its induction has been suggested to represent an important cellular protective response against oxidative damage produced by free heme and hemoglobin (1–5). The HO system catalyzes the degradation of the heme molecule to biliverdin and iron, with the concurrent release of carbon monoxide (CO). Biliverdin is then converted to bilirubin by the cytosolic enzyme, biliverdin reductase. Induction of HO may specifically decrease cellular heme (pro-oxidant) and elevate bilirubin (antioxidant) levels (5–8). Rabbit coronary microvessel endothelial cells, stable transfected with the human HO-1 gene, acquired substantial resistance to toxicity by exposure to high concentrations of hemoglobin as compared with nontransfected cells (9, 10). Two HO isozymes, the products of distinct genes, have been described (11, 12). HO-1, which is ubiquitously distributed in mammalian tissues, is strongly and rapidly induced by many compounds that elicit cell injury; heme, the natural substrate of HO, is itself a potent inducer of the enzyme (11, 12). HO-2, which

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is constitutively expressed, is present in high concentrations in tissues such as brain and testis. Recently, McCoubrey *et al.* (13) described a 33-kDa protein coding for HO-3.

Acute phase proteins and the acute phase response play an important role in the inflammatory process (14, 15). Some of the acute phase proteins are believed to display anti-inflammatory activities because of their antioxidant properties. HO-1 is among the acute phase proteins (2) and is induced within minutes by interleukin-6 (IL-6), IL-1, and endotoxins (16–19). This process involves the transcriptional activation of several regulatory sites in the HO-1 promoter region. AP-1 and IL-6 responsive elements are found in the promoter region of this gene (18, 20). Previous studies from our laboratory have demonstrated that the proximal promoter region of the human HO-1 gene also contains NF-κB and AP-2 binding sequences (21). The finding of AP-2 and NF-κB binding sites on the HO-1 promoter suggests the importance of HO activity in stress/injury responses when these transcriptional factors are known to be activated (21). Dex, on the other hand, prevents accumulation of HO-1 mRNA in the presence of known inducers (19). Dex and other glucocorticoids have been used as anti-inflammatory agents, but they also have dramatic effects on cell proliferation and differentiation (22–27). Lavrosky *et al.* (28) have shown that Dex induces the binding of a transcriptional factor on the NF-κB-like element in the HO-1 promoter.

In this study, we have investigated the role of Dex in heme and IL-6–induced HO-1 gene expression. Due to the strikingly opposite roles of heme and IL-6 as inducers of inflammatory processes and adhesion molecule (29) and glucocorticoids in inflammation (26), we examined the possibility of a functional antagonism between Dex and heme and IL-6 in the activation of the HO-1 promoter region. Our results demonstrated that IL-6–induced upregulation of human HO-1 gene expression can be abolished by Dex following activation of nuclear binding protein to *cis* element transcriptional regulatory site on the STAT3-like responsive element sequence placed between −167 and −158 of the human HO-1 promoter. Furthermore, Dex failed to decrease HO-1 gene expression by heme.

Materials and Methods

Materials. Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco-BRL (Grand Island, NY). Guanidine thiocyanate was from Fluka (Ronkonkoma, NY). SeaKem LE agarose was from FMC Corporation (Rockland, ME). Restriction endonucleases were from Promega Corporation (Madison, WI). Multiprime DNA labeling system, Hybond-N^+ membranes, [α-^{32}P] dCTP were from Amersham Life Science (Arlington Heights, IL). Autoradiographic films were purchased from NEN Dupont (Wilmington, DE). The CAT-ELISA Kit and β-Gal ELISA Kit were obtained from Boehringer-Mannheim (Indianapolis, IN). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was from Clontech (Palo Alto, CA). Stat3, Stat1, and NF-κB consensus oligonucleotides were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Recombinant human interleukin-6 (IL-6) was obtained from R&D Systems Inc. (Minneapolis, MN). Heme and dexamethasone were from Sigma Chemical Company (St. Louis, MO). Heme solutions were prepared fresh each time in the same way as previously described (17).

Cell Culture and Treatment. Rabbit coronary microvessel endothelial cells were obtained from Dr. Mary Gerritsen (30) and cultivated in DMEM supplemented with 10% (v/v) fetal bovine serum at 37°C, in a 5% CO_2 standard humidified incubator. Cells were cultured in fresh medium, and 50 μ*M* Dex, or 10 μM heme or 25 ng/ml IL-6 were added for the time periods indicated.

RNA Extraction and Northern Blot Analysis. Total RNA was isolated according to the method of Chomczynski and Sacchi (31). Cells were washed twice with PBS, and then homogenized in a solution containing 4 *M* guanidine thiocyanate, 25 m*M* sodium citrate pH 7.0, 0.5% *N* laurylsarcosine and 0.1 *M* 2-mercaptoethanol. RNA was extracted first with phenol-chloroform-isoamyl alcohol mixture (50:49:1), followed by an additional extraction with chloroform-isoamyl alcohol (49:1) and a precipitation with ethanol. Total RNA was redissolved in diethyl pyrocarbonate–treated water and quantitated by absorbency measurement using a Beckman DU7400 spectrophotometer. Fifteen micrograms of total RNA were denatured and size-separated by electrophoresis at 100 V on 1.2% agarose gels containing 2.2 *M* formaldehyde and then blotted to Hybond N^+ filter. Prehybridization, hybridization to radiolabeled cDNA probes, and washing steps were performed as described elsewhere (21). Radioactive signals were detected by exposing the filter to x-ray film at −80°C with intensifying screens and quantitated using the Beckman DU 7400 spectrophotometer.

cDNA Probes. For hybridization, the following probes were used: the 883-base pair (bp) *Eco* RI-*Hind* III fragment obtained from a rat HO-1 gene expression vector and a plasmid containing full-length cDNA for rat HO-1 (32). The fragment was obtained by electrophoresis in a 1% agarose gel and excision of the corresponding band and then labeled to high specific radioactivity using [α-^{32}P] dCTP and the multiprime DNA-labeling kit (Amersham) according to the method of Feinberg and Vogelstein (33). As control, all filters were reprobed with a cDNA fragment encoding the G3PDH gene to ensure that equal amounts of RNA were loaded onto each lane.

Assay of HO Activity. Enzyme activity was assessed as described previously (34). Briefly, microsomes prepared from untreated and treated endothelial cells were incubated with 50 μ*M* heme, 2 mg/ml rat liver cytosol, 1 m*M* $MgCl_2$, three units of glucose-6-phosphate dehydrogenase, 1 m*M* glucose-6-phosphate, and 2 m*M* $NADP^+$ in 0.5 ml of 0.1 *M* potassium phosphate buffer, pH 7.4 for 30 min at 37°C in the dark in a shaking water bath. The reaction

was stopped by placing the tubes on ice, and amounts of bilirubin generated were estimated using a scanning spectrophotometer (Perkin-Elmer, Lambda 17 UV/VIS) and defined as the difference between 450 and 530 nm with an extinction coefficient for bilirubin of 40 M cm^{-1}. The wavelength of bilirubin was maximum at 467 nm. Results are expressed as nmol bilirubin/mg protein/hr.

Plasmids and Reporter Gene Activities. The control plasmid pRc/CMV (Invitrogene, CA) and pRc/CMV-s human HO-1, containing the entire protein-coding region of human HO-1 (9) were used for stable transfection. Five plasmids were used for 5′ deletion analysis of the HHO-1 promoter: plasmid (*A*) was constructed by inserting the *Eco*RI-*Xho*I fragment (nucleotide residue −1500 to +19) of the HHO-1 promoter in the skII plasmid (Strategene, San Diego, CA) in the *Eco*RI-*Xho*I sites. Then, the *Bam*HI-*Xho*I fragment containing the human HO-1 promoter (from the construct described above) was inserted into the *Bam*HI-*Xho*I site of tk-CAT plasmid instead of the tk promoter. Other plasmids (*B, C, D, E*) were constructed from plasmid (*A*) by digestion with retraction enzymes, and the ends were filled in using kleenow enzyme and dNTP; the product was digested with *Bam*HI and *Xho*I to obtain (−60 bp to +20 bp). The CMV-gal plasmid (cytomegalovirus promoter region fused to the gal gene) was purchased from Stratagene (San Diego, CA). CAT activity was analyzed as described previously (21, 28). In a typical assay, 30 μl of cell lysate were added to assay buffer containing Tris-HCl (pH 7.9) and 0.15 μCi (^{14}C)-chloramphenicol. After incubation, the reaction mixture was extracted with 500 μl of acetyle acetate, and the acetylated products were separated from (^{14}C)-chloramphenicol by thin-layer chromatography. The ratio of acetylated/total (^{14}C)-chloramphenicol was measured by the aid of a phoshorImager (Molecular Dynamic, Sunnyvale, CA). This ratio was then divided by the galactosidase activity of the same cell lysate to give normalized CAT activity.

Electrophoretic Mobility Shift Assay (EMSA) and Nuclear Protein Isolation. EMSA was performed as described (21, 28). Nuclear protein extracts were prepared from untreated or IL-6–treated cells as previously described (21). A synthetic double-stranded Stat3-oligonucleotide from the human HO-1 promoter to the corresponding 21-bp sequence between −173 and −152 (5′-GACTTTGTTTCCCAAGGGTCA-3′) was used. This oligonucleotide was end-labeled with [γ-^{37}P] ATP and T_4DNA polynucleotide kinase, and purified by polyacrylamide gel electrophoresis. Crude nuclear protein extracts (5 μg) were incubated with labeled double-stranded oligonucleotide (approximately 10,000 cpm) in a reaction buffer containing 20 mM Hepes (pH 8.0), 5 mM dithiothreitol, 1 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 50 mM KCl, 5 mM MgI_2, and 5% (v/v) glycerol. For specificity determinations, nuclear extracts were incubated with a 100-fold molar excess of unlabeled specific (STAT3:5′-GATCCTTCTGGGAATTCCTAGATC-3′) or nonspecific oligonucleotide competitors AP-2 (5′-CGGATGTCCTATTAGGACATCTGCGTCAGCAG3′); NF-κB (5′-AGTTGAGGGACTTTCCCAGGC-3′); and STAT1 (5′-CATGTTATGCATATTCCTGTAAGTG-3′). After 20 min of incubation at room temperature, the reaction products were fractionated by electrophoresis at 4°C in 6% polyacrylamide gels, and the gels were dried down prior to exposure to autoradiographic film.

Statistical Analysis. The data are presented as mean ± standard deviation. Statistical significance of differences between the experimental group and control group was assessed using Student's two-tailed t tests and analysis of variance for the comparison of two treatments. Statistical difference was accepted at $P < 0.05$.

Results

Time-Dependent Accumulation of HO-1 in Endothelial Cells Following Heme Exposure. We performed experiments to evaluate the effect of heme on HO-1 gene expression by measuring the levels of HO-1 mRNA and overall HO activity in endothelial cells. We measured, by Northern blot analysis, the corresponding HO-1 mRNA levels in endothelial cells exposed to heme from 1–6 hr. As shown in Figure 1A, HO-2 mRNA was barely detectable in control cells (Lane 1), and a faint band was apparent after prolonged exposure of Northern blot film. The level of endothelial cell HO-1 mRNA was increased by heme as early as 1 hr (Lane 2) and reached a plateau after 5–6 hr (Fig. 1A, Lanes 6 and 7, respectively). Further, the basal levels of HO-1 mRNA were significantly higher in endothelial cells treated with heme up to 24 hr (Lane 8) as compared to untreated cells (Lane 1).

A quantitative evaluation of the HO-1 mRNA changes by densitometry analysis indicated a 30-fold increase in HO-1 mRNA levels in the endothelial cells treated with heme for 3 hr as compared to cells treated with the vehicle solution (Fig. 1A, Lane 1). We next examined the effect of Dex on heme-induced HO-1 mRNA. Northern blot analysis presented in Figure 1B showed that Dex pretreatment of endothelial cells failed to abolish HO-1 mRNA levels in cells treated with heme for 2–5 hr (Fig. 1B, Lanes 3–6, respectively).

Effect of Dex on Heme-Induced HO Activity. To correlate the appearance of HO-1 mRNA with the time necessary for detectable activity, time-dependent changes in HO activity were measured in response to heme exposure as shown in Figure 2. Cells were incubated with heme for 6 and 24 hr. The basal level of endothelial cell HO activity was 0.63 ± 0.03 nmol bilirubin/mg protein/hr. Treatment with heme for 6 hours increased the enzyme activity to 2.44 ± 0.23 nmol bilirubin/mg protein/hr (a 4-fold increase). After 24 hr incubation with heme, HO activity decreased to 1.87 ± 0.125 nmol bilirubin/mg protein/hr (a 3-fold increase over control). Addition of Dex to heme-treated endothelial cells failed to decrease HO activity significantly.

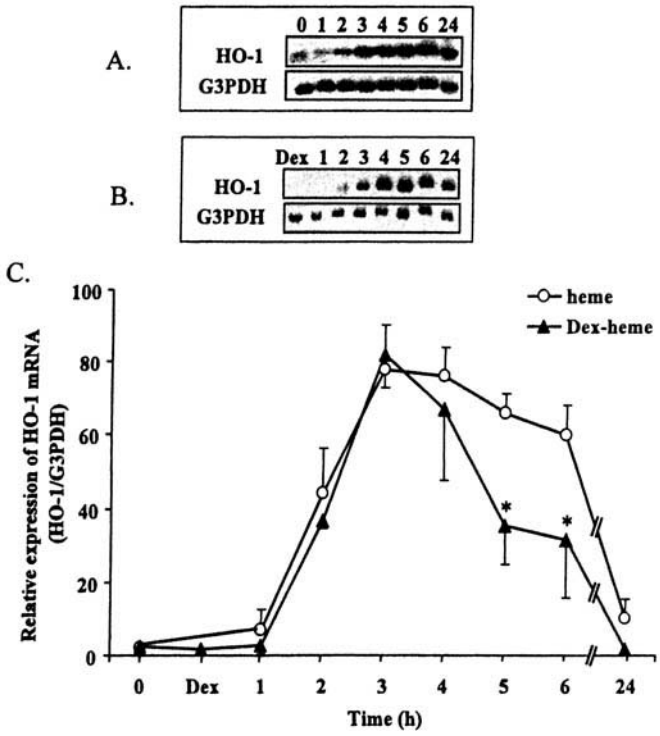

Figure 1. Effect of Dex on heme-induced HO-1 mRNA expression in EC. Confluent cell cultures were incubated with 10 μ*M* heme with or without 50 μ*M* Dex for 1–6 hr, and total RNA was extracted; Lane 1, untreated cells; Lane 2, 1 hr; Lane 3, 2 hr; Lane 4, 3 hr; Lane 5, 4 hr; Lane 6, 5 hr; Lane 7, 6 hr; and Lane 8, 24 hr following heme treatments. Quantitations of HO-1 and G3PDH signals were performed using a densitometry scanner. The relative units correspond to the ratio between the density of the signal obtained with rat HO-1 probe, and the signal obtained with G3PDH. (A) Northern blot analysis of cells treated with heme; (B) Northern blot analysis of cells treated with Dex and heme; (C) relative expression of HO-1 mRNA level compared to G3PDH (○ Heme; ▲ Dex-heme). Results are representative of three different exeriments with similar results. *Significantly different from control.

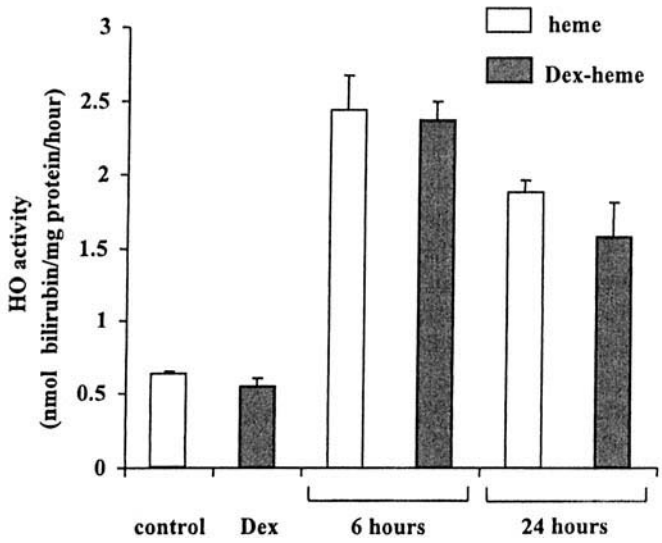

Figure 2. Effect of Dex on heme-induced heme oxygenase activity. Control: untreated cells; Dex: cells treated 3 hr with 50 μ*M* Dex; Heme: cells treated for 6 or 24 hr with 10 μ*M* heme; Dex-heme: cells treated with 50 μ*M* Dex and 10 μ*M* heme for 6 and 24 hr incubation. The enzyme activity was measured as explained in Materials and Methods. Data are expressed as nmol of bilirubin/mg protein/hr and are the result of four determinations.

Differential Effect of Dex on HO-1 Gene Expression in IL-6–Induced Cells. Since IL-6 has been shown to cause rapid activation of HO-1 gene expression and an increase in the levels of HO-1 mRNA, we examined the effect of Dex on IL-6–stimulated HO-1 gene expression. Endothelial cells were preincubated with IL-6 and Dex at the same conditions described in Figure 1. Treatment of cells with IL-6 for 6 hr increased HO activity from 0.6 ± 0.13 nmol bilirubin/mg protein/hr to 1.1 ± 0.24 nmol bilirubin/mg protein/hr. Pretreatment of cells with Dex abolished IL-6–stimulated HO activity (Fig. 3A, upper panel). Similarly, incubation of endothelial cells with IL-6 for 24 hr resulted in a 3-fold increase in HO activity, which was suppressed by pre-incubation with Dex (Fig. 3B, lower panel).

To evaluate the differential effects of Dex on HO isoforms in endothelial cells pretreated with heme or IL-6, total RNA and protein were assessed for HO-1 and HO-2 mRNA levels and HO activity, respectively. We measured, by Northern blot analysis, the corresponding mRNA levels of HO-1 and HO-2 in endothelial cells treated with the various agents for 6 hr. As seen in Figure 4, treatment of endothelial cells with heme or IL-6 stimulated the expression of HO-1 mRNA without affecting the steady state levels of HO-2 mRNA (Lanes 3 and 5). Although HO-1 mRNA appears absent in untreated control cells, a faint signal was apparent after prolonged exposure of the Northern blot film (Fig. 4A, Lane 1). We further examined the effect of Dex on heme- and IL-6–stimulated HO-1 mRNA accumulation in endothelial cells. Having received heme (10 μ*M*) or IL-6 (25 ng/ml) 2 min later, the endothelial cell culture flasks were exposed to Dex at 50 μ*M* for 6 hr. Figure 4A showed that Dex abolished the induction of HO-1 mRNA by IL-6 (Lane 6) but failed to alter the heme effect on accumulation of HO-1 mRNA (Lane 3). Dex alone, on the other hand, did not change HO-1 or HO-2 mRNA levels significantly (Fig. 4A, Lane 4 vs Lane 1).

Figure 4B depicts parallel changes in HO activity following treatment with either IL-6 or heme with and without Dex. The basal levels of HO activity significantly increased following heme treatment (Fig. 4B, Lane 2). This increased activity corresponded to the increased expression of the HO-1, but not HO-2, isoform (Fig. 4A). Dex was unable to suppress heme-stimulated HO activity (Lane 3). Endothelial cells exposed to IL-6 demonstrated a many-fold increase in HO-1 mRNA levels followed by a 2.5-fold increase in HO activity. In contrast to heme, IL-6–induced HO-1 mRNA HO activity were suppressed following exposure to Dex (Fig. 4B, Lane 6). Unlike HO-1, HO-2 mRNA was not induced by heme (Lanes 2 and 3), but we observed a slight increase in HO-2 mRNA after addition of Dex (lanes 5 and 6).

Identification of the Dex Binding Site in Human HO-1 Promoter. The differential effect of Dex on heme and IL-6–stimulated HO-1 gene may be due to preferen-

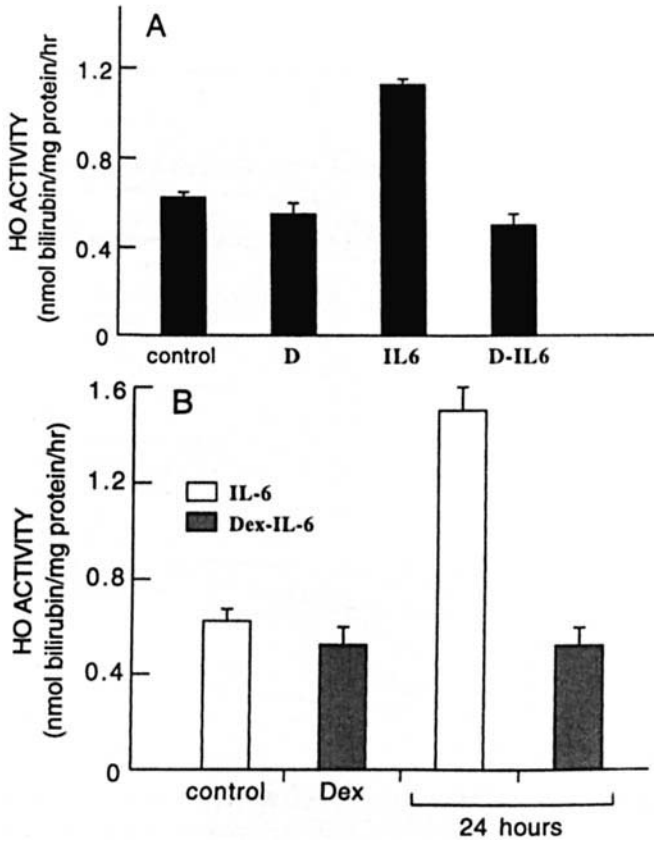

Figure 3. HO activity measurement in endothelial cells treated with IL-6 (Panel A). Control: untreated cells; Dex: cells treated with 50 μ*M* Dex for 6 hr, cells treated with IL-625 ng/ml for 6 hr or cell treated with a combination of Dex and IL-6 for 6 hr. (Panel B) Similar conditions in cells incubated 24 hr with various agents in Panel A. Heme oxygenase was measured as described in Material and Methods.

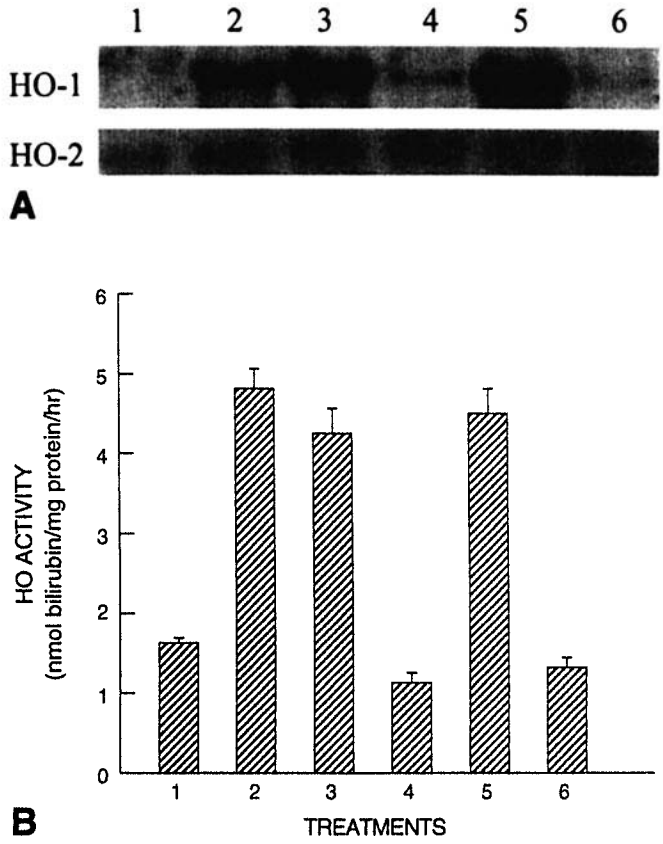

Figure 4. HO isoforms and HO activity measurement in endothelial cells exposed to heme and IL-6 for 6 hr. (Panel A) Northern blot analysis of endothelial cells treated with vehicle buffer, Control cells: Lane 1; cells treated with heme (10 μ*M*): Lane 2; heme (10 μ*M*) and Dex (50 μ*M*): Lane 3; Dex (50 μ*M*): Lane 4; IL-6 (25 ng/ml): Lane 5; IL-6 and Dex: Lane 6 (Panel B) HO activity of endothelial cell treated under the same conditions as in Panel A. Enzyme activity was measured as described in Materials and Methods, and data were expressed as nmol of bilirubin/mg protein/hr.

tially expressed transcriptional factors that block IL-6 activation. A recent study by this laboratory demonstrated that the proximal promoter region of the human HO-1 gene contains AP-2, AP-1, NF-κB, and GRE (21, 28). The finding of AP-1, NF-κB, and GRE suggests that the suppressive effect of Dex on IL-6–induced human HO-1 gene expression may involve specific activation of one of these regulatory sites. Experiments were performed to investigate the basal promoter activity of several human HO-1 promoters using the transient transfection technique and examine the effect of Dex on suppression of human HO-1 in promoters as measured by CAT activity (Fig. 5, left panel). For this purpose, endothelial cells transfected with constructs *A, B, C, D,* and *E* were treated with IL-6 (25 ng/ml). Figure 5 showed that the two constructs *B* and *C,* were the most inducible in cells pretreated with IL-6. The *A* construct had much less potential for inducibility compared with other constructs, suggesting the presence of negative regulation in the upstream region between –280 and –1500. Addition of Dex to endothelial cells transfected with the various constructs resulted in suppression of only two constructs, *B* and *C,* after induction by IL-6. These experiments showed that the promoter region between –120 and –280 in constructs *B* and *C* is responsible for glucocorticoid-directed downregulation of the human HO-1 gene.

Identification of the STAT3 Binding Site. A computer analysis using the sequence of the human HO-1 promoter region (accession number X14782) and the NIH database identified the presence of an IL-6–responsive element (Stat3/APRF) in the constructs *B* and the –166 to –158 (Fig. 6). Therefore, we examined the presence of IL-6–inducible nuclear protein using EMSA assays. For this purpose, we used the ^{37}P-labeled 21-bp oligonucleotide corresponding to the STAT3 sequence found in the human HO-1 promoter and nuclear extracts isolated from endothelial cells treated with IL-6. In initial experiments, the binding specificity to STAT3 was confirmed by EMSA using both specific and nonspecific competitor oligonucleotides (Figs. 7A and 7B). Nuclear extracts from control endothelial cells and from cells exposed to IL-6 were incubated with the labeled 21-bp sequence. Incubation of the nuclear extracts from cells exposed to vehicle solution did not result in binding activity (Fig. 7A, Lane 1). In contrast, incubation of nuclear extract from cells exposed to IL-6 resulted in a marked increase in binding activity in a time-dependent manner (Fig. 6A, Lanes 2–6). The maximum increase in binding activity was seen in nuclear extract at 4 and 6 hr (Fig. 7A, Lanes 5 and 6). In competitive experiments (Fig. 7B), addition of 100-fold excess of unlabeled competitor (STAT1) did not change STAT3 binding activity (Lane 4).

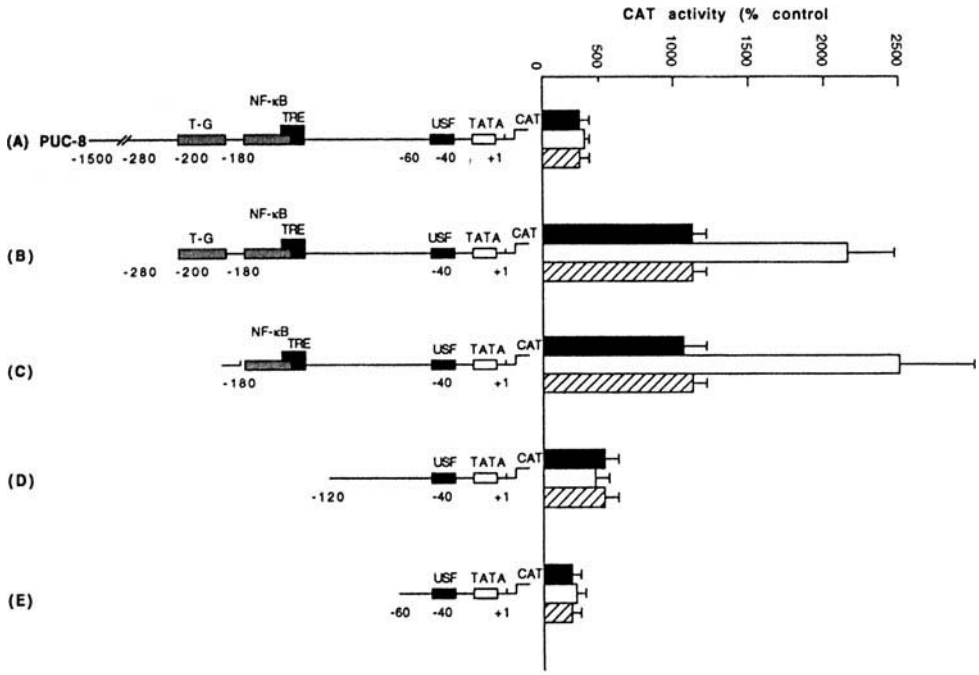

Figure 5. Diagram of the human HO-1 constructs (left panel) and CAT activity (right panel). The cells were dually transfected with pSVβGal and puc-8 constructs. Activity of 5⁻ deletion mutant promoter in endothelial cells was performed as described in Materials and Methods. Briefly, the cells were treated with IL-6, Dex, and control buffer, and 18 hr later, the cells were harvested and reporter gene activities measured. Normalized CAT activities are presented as average of duplicate samples, and the range of values are shown. Constructs used (left panel) were cloned in Puc-8 plasmid as described in Material and Methods; NF-κB, AP-2, TRE, TATA-regulatory elements and potential binding sites are indicated.

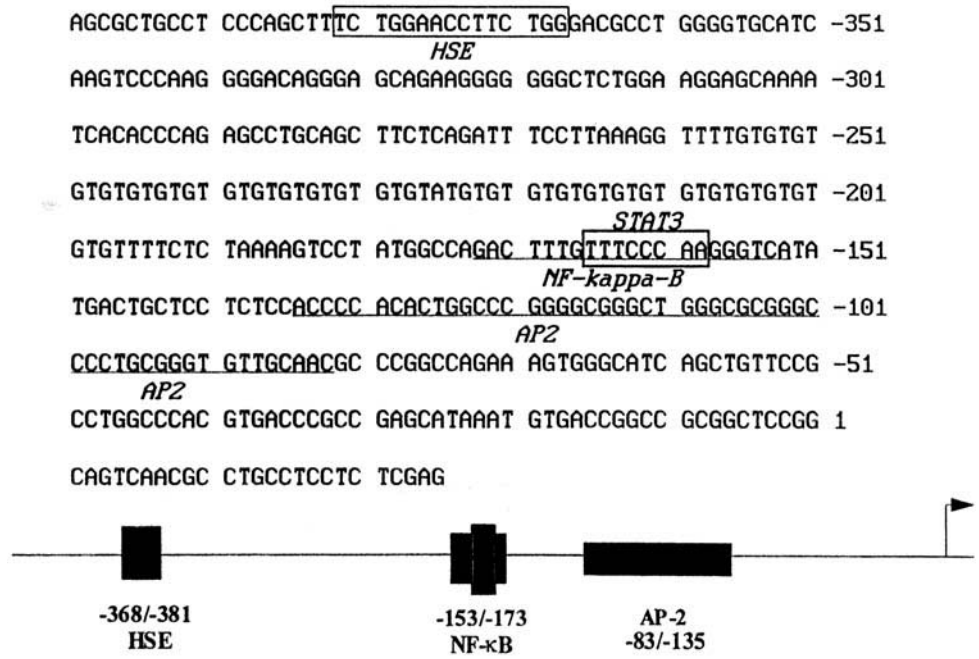

Figure 6. Schematic representation of the human HO-1 promoter. HSE: heat-shock response element, AP-2: AP-2-like element; NF-κB: NF-κB-like element; STAT3: signal transducers and activators of transcription.

By contrast, addition of 100-fold unlabeled specific competitor (consensus STAT3) decreased the binding of nuclear extract protein to 21-bp oligonucleotide (Fig. 7B, Lane 5). Similarly, control endothelial cell extracts did not result in marked binding of STAT3 sequence (Fig. 7B, Lane 1). Furthermore, addition of oligonucleotides containing the consensus sequence for AP-2 was ineffective in decreasing STAT3 nuclear binding activity (Fig. 7B, Lane 6). Addition of oligonucleotides containing the consensus for NF-κB was also ineffective in preventing the binding of nuclear extract to STAT3 (data not shown).

Discussion

The current report describes several novel observations: 1) the induction of HO-1 mRNA and activity by heme is not blocked by the anti-inflammatory agent, Dex (Figs. 1 and 2); 2) addition of heme or IL-6 to endothelial cells increases HO-1, but not HO-2, mRNA; 3) the induction of HO-1 mRNA and the increase in total HO activity in response to IL-6 is blocked by Dex (Figs. 3 and 4); 4) HO-1 promoter CAT assays demonstrated that the upstream region of the human HO-1 promoter (–180 to –120 bp) con-

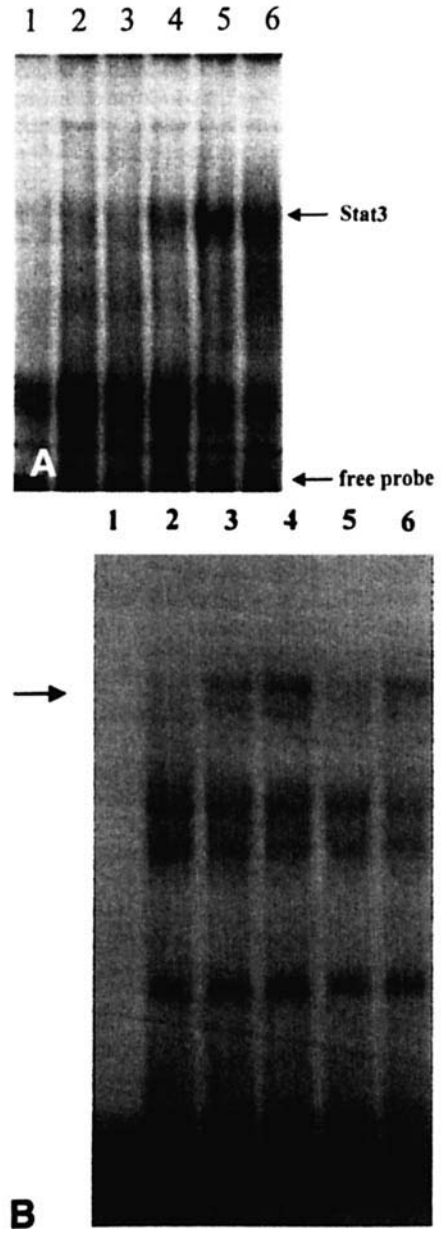

Figure 7. Electrophoretic Mobility Shift Assay. (A) Nuclear extracts from untreated endothelial cells (Lane 1), cells treated with IL-6 25 ng/ml for 30 min (Lane 2), 2 hr (Lane 3), 2 hr (Lane 4), 4 hr (Lane 5), and 6 hr (Lane 6). (B) EMSA using labeled double-stranded STAT3 sequence and nuclear extracts from untreated cells or cells induced 3 hr with 25 ng/ml IL-6. Lane 1: labeled STAT3 probe alone. Lane 2: labeled STAT3 probe incubated with nuclear extracts from untreated cells. Lane 3: labeled STAT3 probe incubated with nuclear extracts from endothelial cells treated with IL-6. Lane 4: labeled STAT3 probe incubated with nuclear extracts from IL-6–induced cells and 100-fold excess of unlabeled competitor (STAT1). Lane 5: labeled STAT3 probe incubated with nuclear extracts from IL-6–induced cells and 100-fold excess of unlabeled STAT3 consensus competitor. Lane 6: labeled STAT3 probe incubated with 100-fold excess of unlabeled AP-2 consensus competitor.

tains the IL-6–responsive element as well as the putative Dex-mediated negative regulator of the IL-6 responses (constructs B and C); and 5) EMSA assay indicated that IL-6 increased the levels of nuclear binding proteins that bind to the consensus STAT3 elements. AP-1 or NF-κB consensus oligonucleotides did not inhibit the increase in IL-6 nuclear binding proteins to STAT3.

The differential effects of heme and IL-6–mediated upregulation of human HO-1 gene expression may be due to repression or activation of different transcriptional factors. Previously, we had identified three regulatory elements, NF-κB, AP-1, and AP-2 elements in human HO-1 promoters (21, 28), which have been implicated in heme-mediated regulation of HO-1 gene expression. Transcriptional control of the HO-1 gene by TPA, H_2O_2, and metals are mainly through activation of NF-κB and AP-1 (20–21, 28, 39–41). The work presented here extends those observations by showing that AP-2 and NF-κB are not involved in the upregulation of HO-1 gene expression by IL-6 treatment. Our finding that IL-6 increases nuclear binding proteins that bind to STAT3 (Fig. 6) supports this notion. Furthermore, we found that AP-2 and NF-κB oligonucleotides do not compete with IL-6 binding proteins to STAT3; although IL-6 responsive elements were also found in the promoter region of the HO-1 gene (36), the nuclear binding protein regulatory sites have not been described. In this paper, we showed that activation of the human HO-1 gene promoter by IL-6 resulted from activation of a promoter region within −185 to −120 bp (Fig. 4). This result is in agreement with the presence of IL-6 inducible nuclear protein at −165 to −158 bp (Fig. 6). Activation or repression of transcription gene expression is largely dependent on the interaction of transcriptional activator and repressor with components of the basal transcription machinery. Several laboratories have shown that NF-κB suppression is a target for steroid and nonsteroid anti-inflammatory agents (37–38). We have reported here that the human HO-1 promoter stimulation by IL-6 involves a regulatory element that appears to be a specific site for STAT3/APRF, independent from NF-κB. The results obtained from the gel retardation assay suggest that induction of endothelial cells by IL-6 lead to the formation of a STAT3 homodimeric complex, which binds to the IL-6–responsive element found in the promoter of human HO-1. This transcription factor is activated by tyrosine phosphorylation *via* the Janus kinase family (22), suggesting that IL-6 activation may involve the JAK-STAT pathway. Although glucocorticoids can affect gene expression by altering protein turnover, RNA splicing, or mRNA stability (24), their most common mode of action in transcriptional regulation of gene transcription (35). The presence of the glucocorticoid-like responsive element in the 5-flank region of the HO-1 gene strengthens the regulatory role of dexamethasone in inflammation. Upregulation of HO-1 gene expression by oxidative stress–inducing agents is mainly through activation of NF-κB (21, 28, 39, 40).

Overexpression of HO-1 by IL-6 and heme may be involved in the pathogenesis of several types of inflammatory reactions and expression of acute stress proteins. Thus, upregulation of HO-1 expression may be beneficial for the control of various types of inflammatory reactions (42).

Several *in vivo* studies on animal models clearly affirm the role of HO-1 as a tissue-protective response to injury and inflammation (43, 44), presumably by elevation of the antioxidants, biliverdin and bilirubin (45). More recently, Poss and Tonegawa (46) have shown that elevation of free radicals in cells deficient in HO-1 gene expression resulted in almost complete cell death. Moreover, it has been reported that overexpression of HO-1 protein by gene transfer (10) or heme could induce angiogenesis (10), a phenomenon closely related to the inflammatory reaction (47). These findings suggest that HO-1 gene expression should be strictly controlled. This notion is supported by the fact that in most types of cells, HO-1 is not constitutively expressed as is HO-2. HO-1 is expressed in response to oxidative stress–inducing agents (for review, see Ref. 1), and it rapidly returns to the basal levels, as continuous expression of HO-1 may be harmful to the cells. Further, da Silva *et al.* (48) demonstrated that pre-induction of HO-1 enhanced cell resistance to oxidative stress; however, long-term expression had a deleterious effect on cell viability. More recently, Dennery *et al.* (49) reported that transient HO-1 overexpression provides protection against oxygen toxicity in lung cells; however, some deleterious effects were observed for chronic HO-1 overexpression. Chronic upregulation of HO-1 by inflammatory cytokines may have negative effects on cellular heme-dependent proteins such as cyclooxygenase and cytochrome P450 isoforms. A decrease in cyclooxygenase activities (PGE_2 and PGI_2, two important vasoactive molecules) was observed in endothelial cells following chronic HO-1 overexpression (50). Thus, suppression of HO-1 by Dex in IL-6–induced inflammation may be beneficial for the control of the possible negative effect of continuous expression of HO-1 genes, as seen in chronic inflammation (44, 48). The elucidation of the exact mechanism by which Dex suppresses HO-1 genes may provide a new direction in the development of anti-inflammatory agents, possibly at the STAT3 binding site.

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